APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: POLYVALENT, PRIMARY HIV-1 GLYCOPROTEIN DNA VACCINES AND VACCINATION METHODS

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CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL983023103US

Date of Deposit December 3, 2003

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Application Serial No. 60/430,732, filed on December 3, 2002, and to U.S. Provisional Patent Application Serial No. 60/503,907, filed on September 17, 2003, the entire contents of both of which are herein incorporated by reference.

TECHNICAL FIELD

The invention relates to methods and compositions for the treatment of acquired immunodeficiency syndrome (AIDS).

BACKGROUND

Human immunodeficiency virus (HIV) is the etiological agent of AIDS. There are two types of HIV currently recognized, HIV-1 and HIV-2. HIV-1 is the predominant form worldwide. The form of HIV-1 that dominates the global epidemic is called the major group of HIV-1. There are three HIV-1 groups, the major group (M group), the outlier group (O group), and the non-M/non-O group (N group). The M group is further divided into at least eleven distinct genetic subtypes which are commonly referred to as clades, A, B, C, D, E, F, G, H, I, J, and K, with more sequences awaiting to be classified. Clade B is the most prevalent in the United States, while clade C is the most prevalent worldwide. Geographic distribution of genetic subtypes is continually changing, and current data offers incomplete estimates.

Approximately 95% of the new HIV infections are occurring in developing countries, thus a vaccine may be the most effective way to control the epidemic. However, developing effective vaccines to prevent HIV infection or neutralize HIV infection has been a difficult challenge to the scientific community. It is a primary goal to develop an HIV vaccine that can effectively elicit specific anti-viral neutralizing antibodies as well as cell-mediated immune responses to prevent infection and control the spread of HIV, with a potential for

considerable breadth of reactivity across genetic clades. The extraordinary degree of genetic diversity of HIV has been problematic for vaccine development.

SUMMARY

The methods and compositions provided herein are based, in part, on the discovery that polyvalent, primary isolate DNA vaccines effectively induce an immune response against HIV (e.g., HIV-1). It has also been discovered that boosts with recombinant HIV protein compositions increase immune responses against HIV in subjects that have been administered a polyvalent, primary isolate DNA vaccine.

In general, the invention features nucleic acid compositions including a plurality of sets of nucleic acid molecules, e.g., DNA plasmids, each nucleic acid molecule encoding a human immunodeficiency virus (HIV), e.g., HIV-1, envelope glycoprotein, wherein each set of nucleic acid molecules encodes a different type of HIV envelope glycoprotein, or comprises a primary isolate sequence from a distinct genetic clade. The nucleic acids can be wild-type sequences or sequences that are 80, 90, 95, 98, or 99 percent identical to wild-type sequences. The encoded proteins can be wild-type sequences, or can include conservation amino acid substitutions, e.g., at 1 in 10, 1 in 20, 1 in 30, or fewer, e.g., at 1, 2, 5, or 10 amino acid locations. In certain embodiments, consensus sequences (based on a collection of different wild-type sequences) can be used.

In various embodiments, the HIV envelope glycoprotein can be any one or more of gp120, gp140, gp160, and gp41. The nucleic acid compositions can further include a set of nucleic acid molecules encoding a HIV gag protein. The envelope glycoproteins can be from a clade of a major (M) group of clades, e.g., the clade can be clade A, B, C, D, E, F, G, H, I, J, or K. In alternative embodiments, the envelope glycoprotein can be from a clade of an outlier (O) group of clades or an N group of clades. The envelope glycoprotein can be an envelope glycoprotein of a Ba-L isolate or a B715 isolate. The clade can be clade C. The envelope glycoprotein can be from a Czm isolate. In certain embodiments, one or more of the sets of nucleic acids can include one or more optimized codons.

In another aspect, the invention includes nucleic acid compositions that include a plurality of sets of nucleic acid molecules, wherein the plurality includes two or more of the following sets: a set of nucleic acid molecules, each encoding a human immunodeficiency

virus (HIV) envelope glycoprotein of clade A; a set of nucleic acid molecules, each encoding a HIV envelope glycoprotein of clade B; a set of nucleic acid molecules, each encoding a HIV envelope glycoprotein of clade C; and a set of nucleic acid molecules, each encoding a HIV envelope glycoprotein of clade E; wherein each set of nucleic acid molecules encodes a primary isolate sequence of the envelope glycoprotein. In certain embodiments, the composition can further include a set of nucleic acid molecules encoding a human immunodeficiency virus (HIV) gag protein, wherein the set encodes a primary isolate sequence of the gag protein, e.g., from clade C, and/or from Czm isolate. The gag protein can also be a gag protein of clade B.

In various embodiments, the composition can contain between 50 μg and 2,500 μg of nucleic acid of each set.

In another aspect, the invention includes a pharmaceutical composition containing one or more of the new compositions described herein and a pharmaceutically acceptable excipient.

The invention also features methods of treating an individual with Acquired Immune Deficiency Syndrome (AIDS), by administering to the individual an amount of the new pharmaceutical compositions sufficient to inhibit disease progression due to human immunodeficiency virus (HIV). In these methods, the mode of administration can be topical administration, oral administration, injection by needle, needle-less jet injection, intradermal administration, intramuscular administration, and gene gun administration. The immune response can be a protective immune response, e.g., a cell-mediated immune response, a humoral immune response, or both.

In certain methods, the new compositions can be administered in combination with a second therapy for HIV infection, e.g., therapy a nucleoside reverse transcriptase inhibitor, therapy with a non-nucleoside reverse transcriptase inhibitor, and/or therapy with a HIV protease inhibitor.

The invention also includes methods of inducing an immune response against human immunodeficiency virus (HIV) or an HIV epitope in a vertebrate mammal by administering to the mammal an amount of the new compositions sufficient to elicit an immune response against HIV or an HIV epitope in the vertebrate mammal. These methods can further include isolating immune cells from the vertebrate mammal; and testing an immune response of the

isolated immune cells in vitro. In these methods, the composition can be administered in multiple doses over an extended period of time, (e.g., over a period of 2, 3, 4 weeks or more, e.g., several months).

The methods can also include administering an adjuvant, boost, or facilitating agent before, during, or after administration of the composition. The vertebrate mammals can be a mouse, a rat, a rabbit, a non-human primate, or a human, e.g., a human infected with, or at risk for infection by, HIV. The mode of administration can be topical administration, oral administration, injection by needle, needle-less jet injection, intramuscular administration, intradermal administration, and gene gun administration.

In another aspect, the invention features isolated protein compositions including a set of isolated human immunodeficiency virus (HIV) envelope glycoprotein molecules, wherein each molecule in the set includes a primary isolate sequence.

The invention also includes protein compositions that include a plurality of sets of isolated human immunodeficiency virus (HIV), e.g., HIV-1, envelope glycoprotein molecules, wherein each molecule in the sets includes a different type of HIV envelope glycoprotein, or a primary isolate sequence from a distinct genetic clade. For example, the envelope glycoprotein of each set can be one or more of gp120, gp140, gp160, and gp41. The clades and isolates can be the same as described herein for the nucleic acid compositions. The protein compositions can be included in pharmaceutical compositions that include a pharmaceutically acceptable excipient.

The invention also features methods of treating an individual with Acquired Immune Deficiency Syndrome (AIDS), by administering to the individual an amount of the new pharmaceutical compositions sufficient to inhibit disease progression due to human immunodeficiency virus (HIV).

In another aspect, the invention includes methods of inducing an immune response against human immunodeficiency virus (HIV) or a HIV epitope in a vertebrate mammal by administering to the mammal one or more of the nucleic acid compositions, and administering to the mammal one or more of the new protein compositions; wherein the nucleic acid composition and the protein composition are administered in amounts sufficient to elicit a detectable immune response against HIV or an HIV epitope in the vertebrate

mammal. One can also isolate immune cells from the vertebrate mammal and test an immune response of the isolated immune cells in vitro.

In these methods, the protein composition can be administered after the nucleic acid composition, e.g., between 4 and 8 weeks after the nucleic acid composition. In addition, a cell-mediated immune response can be tested, a humoral immune response can be tested, and/or a neutralizing humoral response can be tested.

The invention also features kits that include one or more of the new nucleic acid compositions, and instructions for administering the nucleic acid compositions to an individual, e.g., according to one or more of the methods described herein. The kits can also include one or more of the new protein compositions that include a set of isolated human immunodeficiency virus (HIV) envelope glycoprotein molecules. The kits can further include one or more additional sets of isolated HIV envelope glycoproteins, wherein each set is a different type of HIV envelope glycoprotein, or comprises a primary isolate sequence from a distinct genetic clade. In these kits, one or more of the HIV envelope glycoproteins encoded by the nucleic acid molecules of the nucleic acid composition can be of a same type or clade as one or more, or each, of the envelope glycoproteins of the protein composition.

The kits can also include one or more of the new protein compositions that include a set of isolated human immunodeficiency virus (HIV) envelope glycoprotein molecules, wherein each set includes a different type of HIV envelope glycoprotein, or a primary isolate sequence from a distinct genetic clade; and instructions for administration of the composition to an individual that has been administered an HIV vaccine, e.g., a nucleic acid HIV vaccine. The kit can include an excipient, e.g., cyclodextrin, and/or an adjuvant, such as QS-21.

The instructions in the kit can indicate that the nucleic acid composition and/or the protein composition is to be administered to the individual two or more times.

The invention also includes methods of increasing an immune response to HIV in an individual that has been inoculated with an HIV vaccine, by administering to the individual one or more of the new compositions in an amount effective to increase the immune response to HIV relative to a control. For example, the individual can have been inoculated with a nucleic acid HIV vaccine.

A "vaccine" is a composition that induces an immune response in the recipient or host of the vaccine. Methods and compositions described herein cover a nucleic acid, e.g., DNA

plasmid, vaccine that induces humoral (e.g., neutralizing antibody) responses and/or cell-mediated immune response (e.g., cytotoxic T lymphocyte (CTL)) responses in the recipient as protection against current or future HIV (e.g., HIV-1) infection. The vaccine can induce protection against infection upon subsequent challenge with HIV. Protection refers to resistance (e.g., partial resistance) to persistent infection of a host animal with HIV. Neutralizing antibodies generated in the vaccinated host can provide this protection. In other situations, CTL responses can provide this protection. In some situations, both neutralizing antibodies and cell-mediated immune (e.g., CTL) responses provide this protection.

Protective responses can be evaluated by a variety of methods. For example, the generation of neutralizing antibodies against HIV proteins (e.g., envelope glycoproteins, "Env gps"), and the generation of a cell-mediated immune response against HIV proteins can both indicate a protective response. Protective responses also include those responses that result in lower viral loads (e.g., in the blood or in lymphoid organs) in a vaccinated host animal exposed to a given inoculum of virus as compared to a host animal exposed to the inoculum of virus, and that has not been administered the vaccine.

"Polyvalency" and "multivalency" are used interchangeably herein and refer to a feature of a nucleic acid or protein composition, e.g., DNA vaccine or protein boost composition, that encodes or comprises a plurality of different proteins. Each nucleic acid, e.g., plasmid, encodes either a different HIV envelope glycoprotein (Env gp) or Env gp in the form of defective HIV viral particles, or an HIV envelope glycoprotein from different clades, or a combination of these possibilities, allowing for flexibility of this polyvalent nucleic acid, e.g., DNA plasmid, vaccine. As used herein, "envelope glycoproteins" (Env gps) refer not only to isolated Env gps, but also to Env gps in the form of defective viral particles. "3-valent" refers to a composition of three distinct antigens (e.g., an env gene of a clade A isolate, and env gene of a clade B isolate, and an env gene of a clade C isolate). Likewise, "4-valent" and "8-valent" refer to compositions with 4 and 8 unique antigens, respectively.

"DP6-001", "DP6-001 formulation", and "DP6-001 vaccine" refers to a formulation of DNA and protein. The DNA component of DP6-001 is a composition containing codon-optimized nucleic acids that encode five different HIV-1 Env (gp120) antigens and a single Gag antigen. The gp120 antigens are from HIV-1 isolates A, B715, Ba-L, Czm, and E. The Gag antigen is from isolate Czm. The protein component of DP6-001 is a protein

composition containing six different HIV-1 gp120 antigens from HIV-1 isolates A, B715, Ba-L, Czm, and E.

"Primary viral isolate" or "primary isolate" nucleic acid or amino acid sequences refer to nucleic acid or amino acid sequences from the cells or sera of individuals infected with HIV (e.g., HIV-1) rather than from a laboratory strain of HIV. A primary viral isolate is a viral isolate that has been expanded and maintained only in primary human T cells, monocytes, and/or macrophages, and has not been expanded and maintained in cell lines. Thus, a primary isolate differs from what is referred to as a "laboratory strain."

Laboratory strains of HIV have been passaged extensively in the laboratory, in some cases for many years. They may be referred to as TCLA strains, which stands for either tissue culture laboratory adapted strains or T cell line adapted strains. On the other hand, primary viral isolates are collected from the field (e.g., from infected human patients) and expanded or passaged in the laboratory, for example, only for the purpose of determining whether or not growth of the virus is possible, and then subsequently one can obtain the viral sequence. Expansion or passaging of the primary isolates occurs by co-culturing the virus with peripheral blood mononuclear cells, for example, to determine if viral growth can occur. The amount of expansion/passaging is dependent on the particular virus and can vary, but in any case, expansion/passaging is thus considered minimal or limited. This minimal or limited passaging is what differentiates a primary viral isolate from a laboratory strain.

The invention provides several advantages. Because of its polyvalency, the new vaccines are less likely to lose their efficacy due to the high mutation rate of HIV. The nucleic acid vaccines described herein provide many different antigens in the form of sequences from distinct genetic clades and thus single mutations of the infecting virus will not readily decrease the vaccines' effectiveness in recipients. Another advantage the invention provides is the induction of broader immune responses, because the different proteins are encoded by primary viral isolate sequences rather than laboratory strains.

The administration of both polyvalent DNA compositions and protein boosts elicits robust humoral and cell-mediated immune responses. The use of the combinations of compositions described herein provides neutralizing antibody responses. The presence of humoral and cell-mediated responses affords better protection from infection in naïve

individuals. The presence of humoral and cell-mediated immune responses can delay disease progression in individuals that are infected with the virus prior to vaccination.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a table depicting DNA and protein antigens, and the program of administration of DNA and protein antigens in vaccination studies in rabbits.

Figure 2 is a table depicting percent neutralization of primary HIV-1 isolates by sera isolated from rabbits immunized with monovalent and polyvalent vaccines. Sera were isolated after DNA immunization. Animal numbers correspond to the rabbit numbers shown in Figure 1.

Figure 3 is a table depicting percent neutralization of primary HIV-1 isolates by sera isolated from rabbits immunized with monovalent and polyvalent vaccines. Sera were isolated after the first protein boost. Animal numbers correspond to the rabbit numbers shown in Figure 1.

Figure 4 is a table depicting percent neutralization of primary HIV-1 isolates by sera isolated from rabbits immunized with monovalent and polyvalent vaccines. Sera were isolated after two protein boosts. Animal numbers correspond to the rabbit numbers shown in Figure 1.

Figures 5A-5C is a set of graphs depicting percent neutralization by sera from rabbits immunized with monovalent and polyvalent vaccines. "Last DNA" corresponds to assays for

sera taken after the last DNA immunization. "Protein-I" corresponds to assays for sera taken after the first protein immunization.

Figure 6 is a bar graph depicting end titration titers of anti-env IgG responses after DNA priming, as measured by ELISA. ELISA plates were coated with the different primary gp120 antigens as indicated (Ba-L, C1, E, B, A, D, F, or G).

Figures 7A-F are graphs depicting anti-gp120 antibody responses after DNA priming and protein boost in 3-valent gp120 vaccine immunized animal group C4. "Last DNA" refers to sera collected after the 4^{th} DNA immunization. "Protein I" refers to sera collected after one protein boost. "Protein II" refers to sera collected after two protein boosts. Data for sera from rabbits immunized with B, C1, and E DNAs, and boosted with B, C1, and E proteins are presented. Figs. 7A and 7B depict data for sera tested against B Env protein. Figs. 7C and 7D depict data for sera tested against C1 Env protein. Figs. 7E and 7F depict data for sera tested against E Env protein.

Figures 8A-P are graphs depicting anti-gp120 IgG responses against Env antigens included in the protein boost in 8-valent gp120 vaccine immunized animal group C7. Data for sera from rabbits immunized with Ba-L, B, C1, E, A, D, F, and G DNAs, and boosted with B, C1, E, and Ba-L proteins are presented. Figs. 8A and 8B depict data for sera tested against B Env protein. Figs. 8C and 8D depict data for sera tested against Ba-L Env protein. Figs. 8E and 8F depict data for sera tested against C Env protein. Figs. 8G and 8H depict data for sera tested against E Env protein. Figs. 8I and 8J depict data for sera tested against A2 Env protein. Figs. 8K and 8L depict data for sera tested against D Env protein. Figs. 8M and 8N depict data for sera tested against F Env protein. Figs. 8O and 8P depict data for sera tested against G Env protein.

Figure 9 is a graph depicting percent neutralization against HIV-1 89.6-GFP reporter virus with rabbit sera obtained before immunization, after DNA immunization, and after one protein boost. Each group of animals was immunized with a different gp120 formulation as indicated.

Figure 10 is a graph depicting percent neutralization against HIV-1 SF162 with rabbit sera obtained before immunization, after DNA immunization, after one protein boost, and

after two protein boosts. Each group of animals was immunized with a different gp120 formulation as indicated.

Figure 11 is a graph depicting percent neutralization against HIV-1 Ba-L with rabbit sera obtained before immunization, after DNA immunization, after one protein boost, and after two protein boosts. Each group of animals was immunized with a different gp120 formulation as indicated.

Figure 12 is a graph depicting percent neutralization against HIV-1 JRCSF with rabbit sera obtained before immunization, after DNA immunization, after one protein boost, and after two protein boosts. Each group of animals was immunized with a different gp120 formulation as indicated.

Figures 13A-D are a set of graphs depicting percent neutralization of HIV-1 clade A primary isolate DJ263 (Figs. 8A and 8D) and clade C primary isolate TV1 (Figs. 8B and 8C) with rabbit sera after DNA priming and protein boost. Each group of animals was immunized with a different gp120 formulation as indicated.

Figure 14 depicts the amino acid sequences of gp120 from A, Ba-L, B715, Czm, and E isolates.

Figures 15A-E are a set of graphs depicting levels of anti-gp 120 IgG responses after each DNA immunization and after each gp120 protein boost. The coating antigens for ELISA are shown on the top of each figure (clade A, B, Czm, and E for Figs. 15A, 15B, 15C, and 15D, respectively). Times of administration of DNA and protein are depicted with solid and dashed arrows, respectively. "GG" refers to gene gun administration. "IM" refers to intramuscular administration. "ID" refers to intradermal administration.

Figures 16A and 16B are a set of graphs depicting levels of anti-gp120 responses in rabbits immunized with DP6-001 vaccine in which DNA was delivered by an IM route. ELISA reactivity of sera from rabbits immunized with four DNA inoculations (IM) (closed circle) or four DNA (IM) and three protein inoculations (IM) (open circle) is shown. Sera were collected 14 days after last DNA or protein immunization and tested against pooled gp120 from clades B (B715 and Ba-L), C, E and A HIV-1 isolates. Data for males are shown in Fig. 16A. Data for females are shown in Fig. 16B.

Figures 17A and 17B are a set of graphs depicting anti-gp120 response in rabbits immunized with DP6-001 formulation in which DNA was delivered by an ID route. ELISA reactivity of sera from rabbits immunized with four DNA inoculations (ID) (closed circle) or four DNA (ID) and three protein inoculations (IM) (open circle) is shown. Sera were collected 14 days after last DNA or protein immunization and tested against pooled gp120 from clades B (B715 and Ba-L), C, E and A HIV-1 isolates. Data for males are shown in Fig. 17A. Data for females are shown in Fig. 17B.

Figures 18A and 18B are a set of graphs depicting anti-gag responses in rabbits immunized with DP6-001 formulation in which DNA was delivered by an IM route. ELISA reactivity of sera from rabbits immunized with four DNA inoculations (IM) (closed circle) or four DNA (IM) and three protein inoculations (IM) (open circle) is shown. Sera were collected 14 days after last DNA or protein immunization and tested against Gag protein. Data for males are shown in Fig. 18A. Data for females are shown in Fig. 18B.

Figure 19A and 19 B are a set of graphs depicting anti-Gag responses in rabbits immunized with DP6-001 formulation in which DNA delivered by ID route. ELISA reactivity of sera from rabbits immunized with four DNA inoculations (ID) (closed circle) or four DNA (ID) and three protein inoculations (IM) (open circle) is shown. Sera were collected 14 days after last DNA or protein immunization and tested against Gag protein. Data for males are shown in Fig. 19A. Data for females are shown in Fig. 19B.

Figures 20A-E are a set of graphs depicting antibody titers in macaques immunized with polyvalent DNA and gp120 protein. Antibody titers in sera of macaques receiving two (DNA2), three (DNA3) and four (DNA4) DNA immunizations and one (Protein 1) and two (Protein 2) boosts were assayed by ELISA against B715 gp120 (A), Ba-L gp120 (B), Czm gp120 (C), E960 gp120 (D) and Gag (E) proteins. Serum was collected two weeks after each immunization. Antibody titers are based on end point ELISA titers and were obtained from the dilution of immune serum producing two times the optical density at 450 nm compared to the corresponding dilution of serum from a naïve animal.

Figures 21A-21C are a set of graphs depicting percent neutralization of SHIV Ba-L by the serum of macaques immunized with DNA prime and protein boost. Serum from each animal collected after four DNA (DNA 4) and one (protein 1) and two (protein 2) boosts

were assayed for neutralizing activity against $SHIV_{Ba-L}$ isolate in U373 cells. Percent inhibition of infection was based on the degree of infection observed in the presence of immune serum compared to untreated controls.

Figures 22A-22E are a set of graphs depicting serum endpoint ELISA titers in macaques immunized with polyvalent DNA and gp120 Protein. Antibody titers in sera of macaques receiving two (DNA 2), three (DNA 3) and four (DNA 4) DNA immunizations and one (Protein 1) and two (Protein 2) boosts at 5, 9 and 13 weeks post protein boost sera were assayed by ELISA against Ba-L gp120 (A), A gp120 (B), E760 gp120 (C), B715 gp120 (D) and Czm gp120 (E) proteins. Serum was collected two weeks after each immunization and 5, 9 and 13 weeks after the second protein boost. Antibody titers are based on end point ELISA titers and were obtained from the dilution of immune serum producing two times the optical density at 450 nm compared to the corresponding dilution of serum from a naïve animal.

Figures 23A-23C are a set of graphs depicting percent neutralization of SHIV Ba-L by the serum of macaques immunized with DNA prime and protein boost. Serum from each animal collected after four DNA (DNA 4; Fig. 23A) and one (protein 1; Fig. 23B) and two (protein 2; Fig. 23C) boosts were assayed for neutralizing activity against SHIVBa-L isolate in U373 cells. Percent inhibition of infection was based on the degree of infection observed in the presence of immune serum compared to untreated controls.

Figures 24A-24R are a set of graphs depicting numbers of IFN- γ Expressing PBMC from macaques immunized with DNA/Protein formulations, in which PBMC were stimulated with Gag peptides. ELISPOT assays were conducted using PBMC of macaques isolated after the fourth DNA (DNA 4), first (protein 1) and second (protein 2) gp120 protein boosts. Several pools of 15 mer peptides with 11 amino acid overlap from Gag protein from HIV-1HXB2 molecular clone were used for stimulation of PBMC for 18 hrs before the spots were developed and quantitated. Data for ID immunization are shown in Fig. 24A-24I. DNA for IM immunization are shown in Figs. 24J-24R.

Figures 25A and 25B are a set of graphs depicting numbers of IFN- γ Expressing PBMC from macaques immunized with DNA formulations encoding wild type and codon optimized *gag* gene, in which PBMC were stimulated with Gag peptides. Comparison of IFN- γ expressing PBMC as measured by ELISPOT against Gag protein in macaques

immunized with DNA encoding wild type and codon optimized gag gene by intradermal route (Study 1 in Fig. 25A, and Study 2 in Fig. 25B).

Figures 26A-26D are a set of graphs depicting numbers of IFN- γ expressing PBMC from macaques immunized with DNA/Protein formulations, in which the PBMC were stimulated with Clade E and Ba-L Env Peptides. ELISPOT assays were conducted using PBMC of macaques isolated after fourth DNA (DNA 4; Figs. 26A and 26C), and after first protein boost (protein 1; Fig. 26B and 26D). Four pools of 15 mer peptides with 11 amino acid overlap from gp120 proteins from HIV-1_{Ba-L} and clade E isolates were used for stimulation of PBMC for 18 hrs before the spots were developed.

Figures 27-38 are representations of wild-type and codon-optimized DNA sequences of gp120 and gag genes of Czm, Ba-L, B, E, and A HIV-1 isolates.

DETAILED DESCRIPTION

The methods and compositions provided herein are based, in part, on the finding that primary HIV-1 isolates from multiple different genetic subtypes of HIV can be combined to create polyvalent DNA compositions that can induce broad antibody responses (e.g., neutralizing antibody responses) and cell-mediated immune responses (e.g., cytotoxic T lymphocyte (CTL)). The methods and compositions provided herein are also based on the finding that protein boosts, in which HIV proteins from primary isolates can be used to augment immune responses in subjects that have been administered polyvalent DNA compositions. Recent strategies have suffered from only minimal immune protection due to escape from CTL recognition (Barouch et al., 2002, Nature, 415:335-339; Goulder et al., 2001, Nature, 412:334-338; Goulder et al., 1997, Nature Med., 3:212-217). To address this problem, nucleic acid sequences from primary HIV-1 isolates are used to generate polyvalent compositions, thus improving cell-mediated immune responses and decreasing the likelihood of CTL escape by the virus, as well as improving neutralizing antibody response. The new methods provide for flexibility in designing compositions based on combinations of vectors encoding different HIV-1 proteins and combinations of HIV-1 proteins.

The protein boosts can include HIV proteins corresponding to the all of the proteins encoded by DNA administered in prior DNA vaccination steps. Alternatively, a subset of proteins corresponding to the DNA vaccine is administered. For example, if DNA encoding

five different HIV proteins are administered (e.g., Env genes from five different HIV-1 isolates), the subsequent protein boost(s) can include all five of the Env proteins, four of the Env proteins, or fewer.

The DNA and protein compositions can include different genes and proteins from HIV isolates. In some embodiments, Env and Gag antigens are encoded by the DNA compositions, and the Env antigens are included in the protein compositions. Accordingly, provided herein are compositions comprising Env glycoproteins (gps), a combination of vectors encoding Env glycoproteins derived from the sequences of more than one HIV-1 primary isolate (e.g., clade A, B, C, D, E, F, or G), a combination of both different types and different clades, and/or combinations encoding HIV-1 gag proteins.

The DNA and protein compositions can include sequences from isolates of multiple clades, or multiple isolates of a single clade. Different combinations may be used. For example, a DNA composition can include genes encoding an antigen from one clade A isolate, one clade B isolate, one clade C isolate, and one clade E isolate. The composition can further include an antigen of a second clade B isolate.

Coding sequences for primary HIV-1 Env gps can be cloned into nucleic acid, e.g., DNA, vaccine vectors to produce a panel of DNA vaccine plasmids. The HIV envelope is the predominant target of neutralizing antibodies in HIV-infected individuals. Thus, a vaccine encoding Env gps can be used to induce neutralizing antibodies. The primary HIV-1 Env gps include gp120, gp140, gp160, and sgp41. To prepare the new vaccines, these Env gps can be encoded by nucleic acids, e.g., DNA, from primary isolates covering seven genetic clades, A, B, C, D, E, F, and G of the HIV-1 major group. These sequences were isolated from distinct geographic regions: North America, Africa, Asia, and South America. The Env gps can also be encoded by DNA from primary isolates covering other genetic clades of the HIV-1 major group (e.g., H, I, J, and K), genetic clades of the HIV-1 O group, and genetic clades of the HIV-1 N group.

Because of the genetic diversity of HIV, the vaccines based on antigens from laboratory strains of HIV-1, as opposed to primary isolates, have been limited in their ability to generate broad immune responses against the prevalent HIV primary strains (e.g., see Barouch et al., 2002, Nature, 415:335-339; Johnston and Flores, 2001, Curr. Op. In. Pharmac., 1:504-510; and Mascola et al., 1996, J. Infect. Dis.,173:340-348). By combining

multiple nucleic acid molecules (e.g., DNA plasmids) encoding primary isolate proteins (e.g., multiple Env gps) into one polyvalent vaccine, the new vaccines provide a considerable breadth of reactivity across genetic clades. Primary isolate DNA can be directly collected from HIV infected patients, passaged minimally if at all, sequenced, and cloned into multiple DNA vaccine vectors to make a polyvalent vaccine. Minimal passaging may be required to expand the DNA if not enough DNA is available for sequencing. This polyvalent vaccine elicits a broad immune response and broad neutralization against Env gps from the different isolates. The polyvalency decreases the likelihood of low efficacy caused by the constantly changing genetic diversification and mutation of HIV.

Nucleic Acid Vaccines

Vaccines are useful in preventing or reducing infection or disease by inducing immune responses, to an antigen or antigens, in an individual. For example, vaccines can be used prophylactically in naïve individuals, or therapeutically in individuals already infected with HIV. Traditional vaccines, which include inactivated viruses or subunit protein antigen, have had poor immunogenicity, poor cell-mediated immunity induction, safety and stability concerns, and low efficacy. The development of nucleic acid vaccines has proved to be promising.

The new DNA vaccines have the advantage of being more resilient to the rapid evolution and mutation of HIV due to their polyvalency. The new DNA vaccines have the added advantage of being derived from primary isolates, which in combination with their polyvalency can induce broader immune response, namely more effective neutralizing antibodies against HIV (e.g., HIV-1) and/or cell-mediated immune responses (e.g., cytotoxic T lymphocyte (CTL)), thus providing a more effective HIV vaccine. This combination of polyvalency and being derived from primary isolate DNA confers its advantages as a novel vaccine for HIV (e.g., HIV-1).

Nucleic Acid Compositions

Nucleic acid compositions that encode antigens of primary HIV isolates are provided. There are many ways of presenting nucleic acid encoding antigen to a host. DNA vaccines can consist of naked DNA plasmid encoding the antigen. Bacterial vectors, replicon vectors,

live attenuated bacteria, DNA vaccine co-delivery with live attenuated vectors, and viral vectors for expression of heterologous genes also can be used. Bacterial vectors such as BCG and Listeria can also be used. In the case of naked DNA replicon vectors, a mammalian expression plasmid serves as a vehicle for the initial transcription of the replicon. The replicon is amplified within the cytoplasm, resulting in more abundant mRNA encoding the heterologous gene such that initial transfection efficiency may be less important for immunogenicity. Live attenuated viral vectors (e.g., recombinant vaccinia (e.g., modified vaccinia Ankara (MVA), IDT Germany), recombinant adenovirus, avian poxvirus (e.g., canarypox (e.g., ALVAC®, Aventis Pasteur) or fowlpox), poliovirus, and alphavirus virion vectors) have been successful in inducing cell-mediated immune response and can be used as well. The avian poxviruses are defective in mammalian hosts, but can express inserted heterologous genes under early promoters. Recombinant adenovirus and poliovirus vectors can thrive in the gut and so can stimulate efficient mucosal immune responses. Finally, attenuated bacteria can also be used as a vehicle for DNA vaccine delivery. Examples of suitable bacteria include *S. enterica*, *S. tymphimurium*, Listeria, and BCG. The use of mutant bacteria with weak cell walls can aid the exit of DNA plasmids from the bacterium.

DNA uptake can sometimes be improved by the use of the appropriate adjuvants. Synthetic polymers (e.g., polyamino acids, co-polymers of amino acids, saponin, paraffin oil, muramyl dipeptide, Regressin (Vetrepharm, Athens GA), and Avridine) and liposomal formulations can be added as adjuvants to the vaccine formulation to improve DNA stability and DNA uptake by the host cells, and may decrease the dosage required to induce an effective immune response. Regardless of route, adjuvants can be administered before, during, or after administration of the nucleic acid. Not only can the adjuvant increase the uptake of nucleic acid into host cells, it can increase the expression of the antigen from the nucleic acid within the cell, induce antigen presenting cells to infiltrate the region of tissue where the antigen is being expressed, or increase the antigen-specific response provided by lymphocytes.

Nucleic acid uptake can be improved in other ways as well. For example, DNA uptake via IM delivery of vaccine can be improved by the addition of sodium phosphate to the formulation. Increased DNA uptake via IM delivery can also be accomplished by electrotransfer (e.g., applying a series of electrical impulses to muscle immediately after

DNA immunization). Adjuvants which can also be added to the vaccine to improve DNA stability and uptake as well as improve immune induction include water emulsions (e.g., complete and incomplete Freund's adjuvant), oil, *Corynebacterium parvum*, *Bacillus Calmette Guerin*, iron oxide, sodium alginate, aluminum hydroxide, aluminum and calcium salts (i.e., alum), unmethylated CpG motifs, glucan, and dextran sulfate. Coinjection of cytokines, ubiquitin, or costimulatory molecules can also help improve immune induction. The antigens described herein can also be fused with cytokine genes, helper epitopes, ubiquitin, or signal sequences to enhance an immune response. Fusions can also be used to aid in targeting to certain cell types.

The medium in which the DNA vector is introduced should be physiologically acceptable for safety reasons. Suitable pharmaceutical carriers include sterile water, saline, dextrose, glucose, or other buffered solutions (e.g., phosphate buffered saline). Included in the medium can be physiologically acceptable preservatives, stabilizers, diluents, emulsifying agents, pH buffering agents, viscosity enhancing agents, colors, etc.

Once the DNA vaccine is delivered, the nucleic acid molecules (e.g., DNA plasmids) are taken up into host cells, which then express the plasmid DNA as protein. Once expressed, the protein is processed and presented in the context of self-major histocompatibility (MHC) class I and class II molecules. The host then develops an immune response against the DNA-encoded immunogen. To improve the effectiveness of the vaccine, multiple injections can be used for therapy or prophylaxis over extended periods of time. To improve immune induction, a prime-boost strategy can be employed. Priming vaccination with DNA and a different modality for boosting (e.g., live viral vector or protein antigen) has been successful in inducing cell-mediated immunity. The timing between priming and boosting varies and is adjusted for each vaccine.

Administration of DNA Vaccines

The nucleic acid compositions described herein can be administered, or inoculated, to an individual as naked nucleic acid molecules (e.g., naked DNA plasmid) in physiologically compatible solution such as water, saline, Tris-EDTA (TE) buffer, or in phosphate buffered saline (PBS). They can also be administered in the presence of substances (e.g., facilitating agents and adjuvants) that have the capability of promoting nucleic acid uptake or recruiting

immune system cells to the site of inoculation. Adjuvants are described elsewhere herein. Vaccines have many modes and routes of administration. They can be administered intradermally (ID), intramuscularly (IM), and by either route, they can be administered by needle injection, gene gun, or needleless jet injection (e.g., Biojector™ (Bioject Inc., Portland, OR). Other modes of administration include oral, intravenous, intraperitoneal, intrapulmonary, intravitreal, and subcutaneous inoculation. Topical inoculation is also possible, and can be referred to as mucosal vaccination. These include intranasal, ocular, oral, vaginal, or rectal topical routes. Delivery by these topical routes can be by nose drops, eye drops, inhalants, suppositories, or microspheres.

Suitable doses of nucleic acid compositions for humans can range from 1 μg/kg to 1 mg/ kg of total nucleic acid, e.g., from 5 μg/kg- 500 mg/kg of total DNA, 10 μg/kg-250 μg/kg of total DNA, or 10 μg/kg-170 μg/kg of total DNA. In one embodiment, a human subject (18-50 years of age, 45-75 kg) is administered 1.2 mg-7.2 mg of DNA. "Total DNA" and "total nucleic acid" refers to a pool of nucleic acids encoding distinct antigens. For example, a dose of 50 mg of total DNA encoding 5 different Env antigens can have 1 mg of each antigen. DNA vaccines can be administered multiple times, e.g., between two-six times, e.g., three times. In an exemplary method, 100 μg of a DNA composition is administered to a human subject at 0, 4, and 12 weeks (100 μg per administration).

Protein Compositions

Proteins, e.g., isolated proteins, encoding antigens of primary HIV isolates can be administered as "boosts" following vaccination with nucleic acid compositions. Recombinant proteins (e.g., proteins produced by cloning DNA encoding antigens of primary isolates using standard molecular biological techniques) can be one source of isolated proteins for boosting. Proteins used for boosting an individual can include the same sequences as encoded by the DNA vaccines previously administered to the individual, e.g., gp120, gp140, gp160, and/or gp41.

For large-scale production of recombinant HIV proteins, transfectant cell lines are generated (e.g., Chinese Hamster Ovary cell transfectants), and cell lines that stably express the HIV proteins are generated from the transfectants. Lines that overexpress the protein are selected for production. Master and working cell banks of selected cells are maintained.

Proteins are expressed by growing cells in large-scale cultures in protein-free medium. Supernatants of the cells are harvested. Protein is then purified (e.g., using affinity chromatography, ion exchange chromatography, and/or gel filtration chromatography), and tested for purity. Proteins are purified and concentrated using techniques such as gel filtration and ion exchange chromatography. Next, proteins are evaluated for identity, potency, purity, quantity, sterility, the presence of endotoxin, and general safety according to Good Manufacturing Practice (GMP) guidelines. Identity can be determined with ELISA with antibodies specific for the clade of the protein. Potency can be evaluated with ELISA (e.g., reactivity of rabbit sera with the purified protein). Purity can be evaluated with SDS-PAGE and silver stain analyses of the protein, and size-exclusion high-performance liquid chromatography. Quantities can be determined by Coomassie-based assays, spectrophotometric assays, and volume measurements. The quality of protein preparations can be determined by visual inspection and pH measurements. Sterility can be determined by methods described in 21 C.F.R. 610.12. Endotoxin can be determined by Limulus Amebocyte assays. General safety can be determined by methods described in 21 C.F.R. 610.11.

Protein compositions containing an immunogenically effective amount of a recombinant HIV protein, or fragments thereof, can be administered. Suitable compositions can include, for example, lipopeptides (e.g., Vitiello et al., 1995, J. Clin. Invest., 95:341), peptide compositions encapsulated in poly(DL-lactide-co-glycolide) ("PLG") microspheres (see, e.g., Eldridge et al., 1991, Molec. Immunol., 28:287-94; Alonso et al., 1994, Vaccine, 12:299-306; Jones et al., 1995, Vaccine 13:675-81), and peptide compositions contained in immune stimulating complexes (ISCOMS) (see, e.g., Takahashi et al., 1990, Nature 344:873-75; Hu et al., 1998, Clin. Exp. Immunol. 113:235-43).

Useful carriers that can be used with the immunogenic compositions and vaccines described herein are well known, and include, for example, thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly L-lysine, poly L-glutamic acid, influenza, hepatitis B virus core protein, and the like. The compositions and vaccines can contain a physiologically tolerable (i.e., acceptable) diluent such as water, or saline, typically phosphate buffered saline. The compositions and vaccines also typically include an adjuvant. Adjuvants such as QS-21, incomplete Freund's adjuvant, aluminum

phosphate, aluminum hydroxide, or alum, are examples of materials well known in the art. Additionally, CTL responses can be primed by conjugating S proteins (or fragments, derivative or analogs thereof) to lipids, such as tripalmitoyl-S-glycerylcysteinyl-seryl- serine (P_3CSS).

Administration of Protein Compositions

Immunization with a composition containing an HIV protein composition, e.g., via injection, aerosol, oral, transdermal, transmucosal, intrapleural, intrathecal, or other suitable routes, induces the immune system of the host to respond to the composition. In one embodiment, a composition of Env proteins is administered. In one embodiment, a composition of Env and Gag proteins is administered.

An exemplary range for an immunogenic amount of protein composition is 5 μg/kg-500 μg/kg, e.g., 10-100 μg/kg of total protein, with adjuvant. In one embodiment, a dose of 325 μg of a protein composition is administered to a human (18-55 years of age, 45-75 kg). An exemplary program of administration of the protein composition includes a first intramuscular boost 8 weeks after the final nucleic acid immunization, followed by a second intramuscular boost with the protein composition 8 weeks after the first boost.

Kits

Kits comprising the nucleic acid and protein compositions are provided. The kits can include one or more other elements including: instructions for use; other reagents, e.g., a diluent, devices or other materials for preparing the composition for administration; pharmaceutically acceptable carriers; and devices or other materials for administration to a subject. Instructions for use can include instructions for therapeutic application (e.g., DNA vaccination and protein boosting) including suggested dosages and/or modes of administration, e.g., in a human subject, as described herein.

The kit can further contain at least one additional reagent, such as a diagnostic or therapeutic agent, e.g., a diagnostic agent to monitor a response to immune response to the compositions in the subject, or an additional therapeutic agent as described herein (see, e.g., "Combination Therapies," below).

In one embodiment, the kit includes a vial (or other suitable container) containing nucleic acids encoding two, three, four, five, or six distinct HIV Env gps. The kit also includes a second vial containing recombinant HIV Env gps that are the same Env gps as encoded by the nucleic acids in the kit. The kit can include QS-21 adjuvant (50 μg/dose/subject) and cyclodextrin as an excipient (30 mg/subject). The adjuvant and the excipient are formulated with the protein, and can be included in the formulation or packaged separately within the kit.

Combination Therapies

The nucleic acid and protein compositions described herein can be used in methods of treating subjects infected with HIV. The methods of treating these subjects with these compositions can include combination therapies, in which other HIV treatments are administered. For example, a subject undergoing DNA vaccination with protein boosting can be administered anti-retroviral drugs individually, or as Highly Active Antiretroviral Therapy ("HAART"), which refers to therapy with various combinations of nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and HIV protease inhibitors.

Nucleoside reverse transcriptase inhibitors include, e.g., zidovudine (AZT); didanosine (ddI); zalcitabine (ddC); stavudine (d4T); lamivudine (3TC); abacavir (1592U89); adefovir dipivoxil [bis(POM)-PMEA]; lobucavir (BMS-180194); and lodenosine (FddA), 9-(2,3-dideoxy-2-fluoro-b-D-threo-pentofuranosyl)adenine.

Non-nucleoside reverse transcriptase inhibitors include nevirapine (BI-RG-587); delaviradine (BHAP, U-90152); and efavirenz (DMP-266).

Protease inhibitors include saquinavir (Ro 31-8959); ritonavir (ABT-538); indinavir (MK-639); nelfnavir (AG-1343) available under the VIRACEPT™ tradename from Agouron Pharmaceuticals, Inc.; amprenavir (141W94), a non-peptide protease inhibitor, tradename AGENERASE™; and lasinavir (BMS-234475).

The new nucleic acid and protein compositions described herein can enhance the effectiveness of any known AIDS therapies, e.g., by reducing the HIV viral load in the infected patient. The compositions and methods described herein can be used as an adjuct therapy to enhance an infected individual's immune response against the virus.

Evaluating Immune Responses to Vaccinations and Protein Boosts

Advances in the field of immunology have allowed more thorough and sensitive evaluations of cellular responses to candidate HIV vaccines. Such assays as intracellular staining (e.g., flow cytometry) and ELISPOT (an enzyme-linked immunosorbent assay format), allow detecting and counting cells producing cytokines (e.g., TNFα and IFN-γ) in response to antigens. For example, isolation of splenocytes or peripheral blood monocyte cells (PBMCs) from animals or human patients followed by in vitro challenge with HIV epitope such as V3, and finally testing by ELISPOT and/or intracellular cytokine staining (ICS), can determine the potential for a cell-mediated immune response in vaccine recipients. Flow cytometry using tetramers (i.e., molecules consisting of four copies of a given class I molecule bound to their cognate peptide and alkaline phosphatase) allows the enumeration of antigen-specific T cells (e.g., detection of T cells that recognize specific peptides bound to major histocompatibility complex (MHC) class I molecules). A standard chromium release assay can be used to assess cytotoxicity. To assess a cell-mediated immune response to a DNA vaccine, the more traditional approaches of measuring T cell proliferation in response to antigen and CTL-mediated killing of autologous cells expressing HIV epitopes can also be used.

ELISA assays and Western blots can be used to assess humoral immune responses. In particular, ELISA and Western blots can be used to assess antibody binding, antibody neutralizing capability, antibody-mediated fusion inhibition, and antibody-dependent cytotoxicity.

MT-2 Assay – An MT-2 assay can be performed to measure neutralizing antibody responses. Antibody-mediated neutralization of HIV-1 IIIB and MN (a B-clade laboratory strain) can be measured in an MT-2 cell-killing assay as described previously (Montefiori et al., 1988, J. Clin. Microbiol., 26:231-237). HIV-1 IIIB and MN induce the formation of syncytia in MT-2 T cells. The inhibition of the formation of syncytia by the sera shows the activity of neutralizing antibodies present within the sera, induced by vaccination. Briefly, vaccinated test and control sera can be exposed to virally infected cells (e.g., MT-2 T cell line). Neutralization can be measured by staining viable cells (e.g., with Finter's neutral red when cytopathic effects in control wells are about >70% but less than 100%). Percentage

protection can be determined by calculating the difference in absorption (A_{540}) between test wells (cells+virus) and dividing this result by the difference in absorption between cell control wells (cells only) and virus control wells (virus only). Neutralizing titers are then expressed as the reciprocal of the plasma dilution required to protect at least 50% of cells from virus-induced killing.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1: Construction of HIV-1 Envelope Glycoproteins gp120, gp140, and gp41 DNA Vaccines from Clade A-G Primary Isolates

The gene fragments encoding the extracellular portion of HIV-1 primary Env gps, either in the form of gp120 or as a cleavable gp140 with the intact natural cleavage site between gp120 and gp41, were PCR amplified from 9 different primary Env genes representing 7 genetic clades, A to G, of HIV-1 group M (Table 1). A pair of consensus PCR primers was designed and used to amplify nine different gp120 genes: a plus strand primer GP120-p-f1 (p-cttgtgggtcacagtctattatggggtacc) (SEQ ID NO:1) and a minus strand primer GP120-p-b1 (ggtcggatccttactccaccactcttctctttgcc) (SEQ ID NO:2). The consensus primers for amplifying gp140 genes were GP120-p-f1 for plus strand and JAPCR502 (cgacggatccttatgttatgtcaaaccaattccac) (SEQ ID NO:3) for minus strand.

The PCR products with the designed blunt-end at the 5' was further digested by BamHI at the 3' end, and cloned into DNA vaccine vector pJW4303 (Chapman, et al., 1991, Nucleic Acids Res 19:3979-3986; Lu et al., 1996, J Virology 70:3978-3991; Lu et al., 1998, AIDS Res and Hum Retroviruses 14:151-155). The vector pJW4303 was first digested with NheI, followed by treatment with Klenow fragment to blunt the end and then cut again with BamHI. The NheI site was regenerated after ligation with the Env inserts, which are in-frame with the tissue plasminogen activator (tPA) leader sequence in pJW4303.

The DNA vaccine plasmids were named as pJW4303/gp120 or pJW4303/gp140.A1, A2, B, C1, C2, D, E, F and G respectively (Table 1). Table 1 shows useful polyvalent vaccine components, the strain and Genebank Accession number from which they were

derived, the genetic clade, and the country of origin. For soluble gp41 (sgp41) DNA vaccines, consensus primers GP41-P-F1 (gtcgctccgctagcgcagtgggaataggagctgtgttccttgggttc) (SEQ ID NO:4) and JAPCR502 were used to amplify sgp41 genes, which were cloned into pJW4303 NheI and BamHI sites. For a given polyvalent vaccine, any combination of the two or more of the components listed in Table 1 can be used. The polyvalent vaccine can be administered as naked DNA plasmid, with a facilitating agent, with an adjuvant, and/or with a protein boost described herein.

Table 1. Polyvalent HIV-1 envelope glycoprotein DNA vaccine components

Polyvalent gp120 DNA vaccine components	Polyvalent gp140 DNA vaccine components	Polyvalent gp41 DNA vaccine components	HIV-1 strains	Genetic clades	Geographic regions	GeneBank Accession number
gp120.A1	gp140.A1	sgp41.A1	92RW020.5	A	Africa	U08794
gp120.A2	gp140.A2	sgp41.A2	92UG037.8	A	Africa	U09127
gp120.B	gp140.B	sgp41.B	92US715.6	B	North America	U08451
gp120.C1	gp140.C1	sgp41.C1	92BR025.9	C	South America	U09126
gp120.C2	gp140.C2	sgp41.C2	93MW965.26	C	Africa	U08455
gp120.D	gp140.D	sgp41.D	92UG021.16	D	Africa	U27399
gp120.E	gp140.E	sgp41.E	93TH976.17	E	Asia	U08458
gp120.F	gp140.F	sgp41.F	93BR020.17	F	South America	U27401
gp120.G	gp140.G	sgp41.G	92UG975.10	G	Africa	U27426

Example 2: Immune Response Raised by HIV-1 Primary Isolate DNA Vaccine

DNA Immunization. A female New Zealand Rabbit (2 kg) received three monthly DNA immunizations by gene gun. Each shot delivered 1 μg of DNA and a total of 36 non-overlapping shots were delivered to each rabbit at each of the three time points at the surface of shaved abdominal skin after animals were anesthetized according to IACUC approved protocols. The serum samples were collected immediately before, and 4 weeks after each immunization.

ELISA (enzyme-linked immunosorbent assay). Rabbit sera samples were tested for gp120-specific IgG antibody responses by ELISA. Microtiter plates were coated with ConA (5 μg per well) for 1 hour and then washed 5 times with washing buffer (PBS at pH 7.2 with 0.1% Triton X-100). Env antigens at 1 μg/ml were added (100 μl for each well) and

incubated for 1 hour at room temperature. Blocking was done with 200 µl/well of 4% milk-whey blocking buffer for 1 hour at room temperature. After removal of the blocking buffer and another 5 time washes, 100 µl of serially diluted sera were added and incubated for 1 hour. The plates were washed 5 times and incubated with 100 µl of biotinylated anti-rabbit IgG diluted at 1:1000 for 1 hour followed with washes. Then, horseradish peroxidase-conjugated streptavidin diluted at 1:2000 was added (100 µl/well) and incubated for 1 hour. After the final washes, 100 µl of fresh TMB substrate was added per well and incubated for 3.5 min. The reaction was stopped by adding 25 µl of 2 M H_2SO_4, and the optical density (OD) of the plate was measured at 450 nm. ELISA assays in which sera reactivity to gp120 was evaluated are described in examples below.

Western blot analysis. The gp120 antigens transiently expressed from 293T-cell supernatants and cell lysates were subjected to denaturing SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membrane. Blocking was done with 0.1% I-Block. Rabbit serum immunized with mixed polyvalent gp120 DNA vaccines was used as the detecting antibody at 1:500 dilution and incubated for 45 minutes. Subsequently, the membranes were washed with blocking buffer and then reacted with AP-conjugated goat anti-rabbit or human IgG at 1:5000 dilution. After final wash, Western-light substrate was applied to the membranes for 5 minutes. Once the membranes were dry, Kodak films were exposed to the membrane and developed with an X-Omat processor. Env reactivity was also observed by Western blot.

Example 3: Neutralization Assay

One way of determining the potential efficacy of a vaccine in animals is to perform in vitro functional assays of the animal's immune cells. The peripheral blood mononuclear cell (PBMC) assay and the MT-2 assay described above are examples of evaluating humoral responses in vaccinated test animals in vitro. As described below, cell-mediated immune responses can also be tested to evaluate the functional ability of immune cells of vaccinated animals. These assays can also be performed in vitro with immune cells isolated from human subjects in determining the potential efficacy of a vaccine.

PBMC Assay. The presence of neutralizing antibodies in the serum of a vaccinated animal was tested in a functional assay referred to as a neutralization assay. Rabbits were

immunized as described in example 2, above, with a monovalent vaccine (rows 3-10) or polyvalent vaccine (row 11). The left column designates with which primary isolate vaccine the rabbit was vaccinated. The sera, collected four weeks after the third immunization, were applied to peripheral blood monocyte cells (PBMCs) infected with different primary viral isolates (designated in the top row). Table 2 shows the results of this PBMC neutralization assay of monovalent and polyvalent immunization and immunization of rabbits. Results from this assay are expressed as percent inhibition of virus as compared with the virus control without immunized rabbit sera. Results from the monovalent vaccinations show a general trend towards the ability to autologously respond. However, as seen in the last row, in which the polyvalent vaccine was used, greater than 57% inhibition against any of the tested primary viral isolates virus was obtained showing that the polyvalent primary HIV-1 Env vaccine was able to generate broad neutralizing antibody responses in the rabbits receiving this vaccine.

Table 2. Neutralization Assay Results

	PBMC with primary isolates			
Rabbit sera	A1	B	C1	E
A1	< 10	38%	100%	<10
A2	99%	< 10	5%	96%
B	< 10	94%	24%	< 10
C1	< 10	< 10	< 10	< 10
C2	< 10	< 10	100%	< 10
D	< 10	97%	100%	< 10
E	32%	58%	13%	93%
F	27%	56%	36%	37%
G	< 10	0%	1%	< 10
A to G	78%	57%	60%	97%

Example 4: Assaying Protective Immunity

The efficacy of the new DNA vaccines can be tested in an animal model. Preferably, responses in animals that can be infected by HIV are tested, such as a non-human primate (e.g., a chimpanzee) or an animal, such as a mouse, which has circulating human immune cells. Large enough numbers of animals should be used to achieve statistical significance, though in the case of non-human primates, the numbers may be limited and thus the

experiments may be repeated in the same animal for example. Once the test animals are vaccinated and control animals are vaccinated with a negative control containing the same vector, but without the heterologous Env gp DNA, both groups of animals can then be infected with HIV. They may be infected with primary isolates or with laboratory strains, or both. After a suitable amount of time to allow infection with HIV and at which the animals vaccinated with negative control vaccine begin to show a decline in T cell number, then the test and control animals can be tested for protective immune response.

One way to test a protective immune response is to obtain sera from the animals and use ELISA (see above) to test for the presence of specific IgG antibody responses (see Example 2 above). The animals can be monitored for the presence, delay or absence of HIV infection relative to negative control animals, using methods known in the art.

Efficacy of a vaccine can be evaluated in uninfected animals by performing *in vitro* functional assays on the immune cells of the vaccinated animal. The presence of neutralizing antibody can be tested in vaccinated animals (e.g., mice, rats, rabbits, non-human primates), which have not been infected with HIV. This neutralization assay is described above in Example 3. Cell-mediated immune responses (e.g., CTL) responses can be tested in animals (e.g., mouse or non-human primate) without infection with HIV. To test a cell-mediated immune response, splenocytes can be isolated. The splenocytes are then exposed to the peptide antigen V3, a commonly used HIV antigen that provides a good epitope to test ability of T cells to mount a cell-mediated immune response *in vitro*. ELISPOT and/or Intracellular Cytokine Staining (ICS) are then performed to determine T cell function. Other tests for ability to resist infection can be performed which are known in the art. Although the best test of protection against HIV is to challenge that animal with HIV, currently there is no definitive way to infect a non-human animal with HIV. SHIV infection of non-human primates has been tested. The current standard of testing in animals to test vaccination is, as discussed, isolation of immune cells of a vaccinated animal and functional testing for activity against antigen such as V3, for example, or generation of neutralizing antibody.

Experiments in which DNA vaccines and protein boosts are tested are described below.

Example 5: Anti-gp120 DNA Vaccination and boosting in rabbits

DNA and protein compositions were prepared with antigens listed in Table 3. The antigens were administered to rabbits according to the study design presented in Figure 1. Briefly, rabbits were immunized with monovalent, 3-valent, 8-valent, or control DNA vaccines at 0, 4, 8, and 16 weeks as listed in Figure 1. Animals received protein boosts at week 24 and 28 as depicted in Figure 1. Neutralization of primary HIV-1 isolates by sera from immunized animals was measured. Neutralization titers for sera collected after the last DNA immunization (and before the first protein immunization) are depicted in Figure 2. Neutralization titers for sera collected after the first protein immunization are depicted in Figure 3. Percentages of neutralization for sera (1:5 dilution) collected after the second protein immunization are depicted in Figure 4. Titers are calculated based on the dilution of immune serum inhibiting 50% of infection as compared to untreated controls. The lower rows of each table under "positive antibodies" list neutralization values obtained with antibodies HIVIG, 2F5, and 2G12, which are known to neutralize in these assays. Sera measurements showing a high level of neutralizing activity are shaded in each figures. Fig. 2 shows that monovalent and polyvalent DNA vaccination resulted in high levels (50%$^{+}$) of neutralizing activity against clade B isolate SF162.

Neutralizing activity to other clade B, C, A, and E isolates was also detected. Fig. 3 shows that DNA vaccination with one protein boost induced high levels of neutralizing activity against clade B SF162, Ba-L, and JRCSF isolates, with low levels of activity against other isolates. Activity was observed in all of the animals receiving polyvalent and approximately half of the animals receiving monovalent vaccination. Both monovalent and polyvalent regimes produced responses with high levels of activity in some animals. Figure 4 shows that the second boost resulted in high neutralizing activity in all animals, with varying degrees of responsiveness to different isolates.

Neutralizing responses were tested against additional clade B viruses (MN, HXB2-GFP, and 89.6 GFP). Construction of viruses expressing green fluorescent protein (GFP) is described in Example 9, below. Figure 5A, B, and C, shows % neutralization observed for sera from the animals listed in the table in Figure 1. Sera taken from animals after DNA vaccination and after one protein boost shows that high levels of neutralizing activity were

induced against isolate MN in animals receiving both monovalent and polyvalent DNA. Neutralizing activity against HXB2 and 89.6 isolates was lower, but high levels were achieved in some animals with protein boosting. Monoclonal antibodies 2F5 and 2G12 neutralized with high levels (75%) of neutralizing activity against those strains in this assay (data not shown).

Table 3: gp120 Immunogens used in DNA Vaccination and Protein Boosting Studies in Rabbits

gp120 immunogen	Genetic subtype	HIV-1 strain	Co-receptor usage	GenBank® Accession No.
A-120	A	92UG037.8	CCR5	U09127
B-120*	B	92US715.6	CCR5	U08451
C1-120*	C	92BR025.9	CCR5	U09126
D-120	D	92UG021.16	CXCR4	U27399
E-120*	EA	93TH976.17	CCR5	U08458
F-120	F	93BR020.17	CXCR4	U27401
G-120	G	92UG975.10	CCR5	U27426
Ba-L-120*	B	Ba-L	CCR5	M68893

Example 6: An HIV-1 Gag DNA Vaccine is Immunogenic in Mice

Cell mediated immune (CMI) responses elicited by Codon optimized Gag DNA vaccines with or without tPA leader sequence were examined.

Balb/C mice (female) were immunized using a gene gun to evaluate the immunogenicity of DNA vaccines. This vaccine included a codon optimized, *gag* gene insert from the HIV-1 isolate Czm. Each animal received 4 monthly immunizations with 6 μg of DNA delivered at each immunization. One week after the last immunization, animals were sacrificed and spleens were collected for analysis of CMI responses. This study compared the relative immunogenicity of two different constructs with a *Gag* gene insert (Table 4). One construct (.wt) used a codon optimized *gag* gene sequence without any additional modification on the coding amino acids. The other (.tPA) codes for an additional human tissue plasminogen leader sequence (tPA) at the very N-terminus end. The tPA leader was reported to be responsible for improved expression and immunogenicity of *Gag* DNA vaccines (Qiu, et al., 2000, J. Virology. 74(13):5997-6005). However, in our previous studies

using non-codon optimized *gag* gene from a laboratory adapted HIV-1 isolate (IIIB), it was found that the wild type *gag* gene insert was more immunogenic than the *gag* gene insert with a tPA leader in inducing CMI responses. This study was undertaken to investigate whether the codon optimized *gag* gene inserts from HIV primary isolate 96ZM651 (Czm) would show similar findings. CMI responses elicited by these vaccines were analyzed by ELISPOT assays and intracellular cytokine (IFNγ) staining (ICS) with an imunodominant peptide from Gag p24 antigen stimulation.

Both ELISPOT and intracellular cytokine staining (ICS) methods were used to measure the cell mediated immune responses. The results are shown in Table 4. Animals that received either of the two *gag* DNA vaccines all showed Gag specific CMI responses, as shown by both ELISPOT and ICS data. The vector alone groups did not show significant Gag-specific CMI responses. The wild type *gag* insert appeared slightly more immunogenic than the *gag* insert with a tPA leader.

Table 4: Average Gag-specific Cell-Mediated Immune Responses in Mice Immunized with Gag DNA Vaccines

Animal groups	No. of animals per group	ELISPOT: Gag peptide* specific spots/million cells	ICS: Gag peptide* specific IFN expressing CD8+ T cells (%)
pSW3891 vector	5	4.25 ± 4.34	0.2 ± 0.03
pSW3891/Gag.Czm. Opt.wt	4	1020 ± 470.31	3.74 ± 2.59
pSW3891/Gag.Czm. Opt.tPA	4	604 ± 538.63	2.29 ± 2.87
* Gag p24 peptide (199-207): AMQMLKDTI; Reference peptide sequence, p24 (aa 65-73)			

Thus, the codon optimized Czm *gag* insert was highly immunogenic in the pSW3891 vector, and it was more immunogenic when expressed with its natural N-terminal sequences than with a tPA leader sequence. This is the design of the construct used in DP6-001.

Example 7: Monovalent and 4-valent Env Vaccines Induce Anti-HIV-1 Env IgG Responses in Rabbits

The immunogenecity of HIV-1 Env DNA vaccine as either monovalent or 4-valent formulations was examined. In this study, New Zealand White (NZW) rabbits (female) were

immunized with DNA vaccines expressing primary HIV-1 Env antigens. Each group included two (Groups 10, 11, 14 and 17) or three rabbits (Groups 1 and 3). Each animal received four monthly DNA immunizations with 36 μg of total DNA plasmid (36 μg of single DNA for the monovalent group and 9 μg of each DNA for the 4-valent group) delivered by a gene gun (Bio-Rad) at each immunization. In this study, non-codon optimized DNA vaccines were used. Sera collected two weeks after the last DNA immunization were measured by ELISA for the levels of anti-Env IgG responses. ELISA plates were coated with recombinant primary Env antigens. The starting serum dilution was 1:500 in these experiments.

ELISA data on NZW rabbit sera induced by either one HIV-1 *Env* DNA vaccine component (monovalent) or a combination of four *Env* DNA vaccine components (4-valent) is summarized in Table 5. While the monovalent sera in general had higher titers against the respective autologous Env antigens than the heterologous Env antigens, the 4-valent DNA vaccine was able to generate a high titer antibody against a broad spectrum of primary HIV Env antigens, including both homologous (such B, Ba-L, Czm and E) and heterologous antigens (such as A2, D, F and G).

Thus, DNA immunization was effective in inducing high titer anti-Env antibody responses with both monovalent and polyvalent vaccine formulations. Polyvalent formulations were able to induce a higher level of antibody responses against multiple primary HIV-1 Env antigens than the monovalent formulation.

Table 5: End Point Titration Titers of Anti-Env IgG of Rabbit Sera

NZW Rabbit Groups	DNA Vaccines Used for Rabbit Immunization	Env Antigens Coated for ELISA Plates							
		Ba-L	B	C	E	A2	D	F	G
1	Ba-L	233,644	54,000	112,325	54,000	162,000	77,881	25,960	77,881
11	B	54,000	324,000	162,000	54,000	54,000	54,000	54,000	36,000
14	Czm	12,000	36,000	486,000	108,000	108,000	54,000	54,000	36,000
3	E	54,000	12,481	112,325	486,000	77,881	37,442	112,325	112,325
17	B, Ba-L, Czm, E	*972,000*	*324,000*	162,000	*486,000*	*486,000*	*108,000*	*324,000*	*324,000*
10	vector	<500	<500	<500	<500	<500	<500	<500	<500

Example 8: Anti-HIV-1 Env IgG Responses for 3-valent and 8-valent DNA + Protein Vaccination

The immunogenicity of polyvalent Env DNA + protein vaccines in rabbits was evaluated. New Zealand White rabbits (female) were immunized with DNA vaccines expressing primary HIV-1 Env antigens. Each group included three rabbits. Each animal received four monthly DNA immunizations with 36 μg of DNA plasmid (12 μg of each DNA for 3-valent group and 4.5 μg of each DNA for 8 valent group) delivered by a gene gun (Bio-Rad) at each immunization. In this study, non-codon optimized DNA vaccines were used.

After two months rest, two monthly protein boosts were administered subcutaneously in Freud's incomplete adjuvant (IFA). The protein boosts matched the DNA priming with the gp120 antigens from the same primary viral isolates (Table 6). Each rabbit received a total of 100 μg of recombinant gp120 proteins for each protein boost (33.3 μg of each protein in Group C4 and 25 μg of each protein in Group C7).

Table 6: Design of Animal Groups for DNA Prime + Protein Boost

Animal Groups	Animal Numbers	DNA Priming Components	Protein Boosting Components
C4	C4-1, C4-2, C4-3	B, C1, E	B, C1, E
C7	C7-1, C7-2, C7-3	Ba-L, B, C1, E, A, D, F and G	B, C1, E, Ba-L

Sera collected two weeks after the last DNA immunization and after each protein boost were measured by ELISA for the levels of anti-Env IgG responses.

To measure anti-Env responses, different primary gp120 antigens were coated individually on the ELISA plates as indicated (Ba-L, C1, E, B, A, D, F or G). ELISA data on NZW rabbit sera induced by either three HIV-1 *Env* DNA vaccine component (3-valent, B, C1 and E) or eight *Env* DNA vaccine components (8-valent, B, C1, E, Ba-L, A, D, F and G) are summarized in Fig. 6. Both 3-valent and 8-valent DNA formulations induced high titer antibody responses against primary HIV-1 Env antigens. The 3-valent formulation elicited high titer antibody responses against both homologous (such B, C1 and E) and heterologous Env antigens (such as Ba-L, A, D, F and G). Under the conditions used, the 8-valent formulation did not appear to improve this immune response.

Anti-Env IgG response after protein boosts in animals which received 3-valent Env DNA vaccine formulation were measured (Fig. 7). In this study, three sera dilutions were tested: 1:10,000; 1:40,000 and 1:160,000. In the "Last DNA" group, sera were collected after the fourth DNA immunization. In the "Protein I" group, sera were collected after one protein boost. In the "Protein II" group, sera were collected after two protein boost. After one protein boost (Protein-I), anti-Env IgG responses in rabbits primed with the same DNA vaccines quickly reached the peak level. The second protein boost (Protein-II) did not increase the response in this experiment. This was true for all three Env antigens (B, C1 and E) tested in this ELISA study.

However, for animals that received 8-valent Env DNA vaccines, two protein boosts were usually required for anti-Env IgG to reach the same level as found in the 3-valent group. The data in Fig. 8A depict the rabbit IgG responses against primary Env antigens included in both DNA prime and protein boost immunizations. The data in Fig. 8B depict the IgG responses against primary Env antigens that were included only in the DNA priming phase. The patterns were very similar between the groups. Two proteins were needed to induce a higher level of anti-Env IgG responses.

These data show that DNA immunization with a 3-valent HIV-1 Env formulation induced an effective immune response against gp20 from homologous and some heterologous strains of HIV-1. This response was not improved with the use of an 8-valent formulation, under the conditions used for these studies. Recombinant HIV-1 Env proteins were very effective in boosting the anti-ENV responses in all DNA-primed animals. One protein boost was needed to reach peak antibody levels in animals receiving 3-valent DNA priming while two protein boosts were needed with the 8-valent group to reach the same peak levels. In DP6-001, five primary Env DNA vaccines are included in the priming phase to cover at least 4 clades of HIV-1 Env antigens without compromising the immunogenicity of DNA priming. Two protein boosts are proposed in DP6-001 to maximize the boosting effect.

Example 9: Neutralizing Antibody Responses with Rabbit Sera Immunized with Monovalent and Polyvalent Env DNA + Protein Formulations

The neutralizing antibody responses elicited by DNA prime + protein boost vaccine regiment against multiple primary HIV-1 isolates was examined. As shown in Table 7, each

animal received 4 monthly DNA immunizations (DNA priming) and two monthly protein boosts at varying individual dose as described below. For mono-valent and 3-valent groups, the protein boosts matched the DNA priming with the same primary gp120 antigens. The monovalent group (Group C1) received gp120.Ba-L, a primary HIV-1 Env antigen. The 3-valent group (Group C4) received three primary HIV-1 Env antigens: gp120-B, gp120-C1 and gp120-E. For 8-valent group (Group C7), animals received DNA vaccines expressing eight primary HIV-1 Env antigens: gp120.A, gp120-B, gp120-Ba-L, gp120-C, gp120-D, gp120-E, gp120-F and gp120-G followed by the protein boosts including 4 recombinant gp120 antigens: gp120-B, gp120-Ba-L, gp120-C and gp120-E. Group C10 is a control group in which rabbits were first inoculated four times with the empty DNA vector pSW3891, followed with protein boost of a mixture of four Env protein antigens (same as Group 7). In this pre-clinical study, the *env* genes inserted in the DNA vaccines were not codon optimized.

Table 7: Design of Animal Groups for HIV-1 Neutralizing Antibody Responses

Animal Groups	Animal Numbers	gp120 DNA Priming Components	gp120 Protein Boosting Components
C1	C1-1B, C1-2B	Ba-L	Ba-L
C11	C11-1, C11-2	B	B
C2	C2-1, C2-1	C1	C1
C3	C3-1, C3-2	E	E
C4	C4-1, C4-2	B, C1, E	B, C1, E
C7	C7-1, C7-2	Ba-L, B, C1, E, A, D, F and G	B, C1, E, Ba-L
C10	C10-1	Vector control	B, C1, E, Ba-L

Rabbit sera from animals described in Table 7 were collected at pre-immunization, after the fourth DNA immunization, and after the first and second protein boosts. Neutralization assays were conducted to examine whether rabbit sera with positive anti-Env IgG antibody responses could neutralize primary HIV-1 isolates. The neutralization activity of each serum was tested at 1:5 dilution using a green fluorescent protein (GFP) indicator assay system.

Recombinant green fluorescent protein (GFP) reporter viruses for use in the neutralization assays were generated by co-transfection of 293T cells with the pNL4-3env- plasmid (full-length NL4-3 HIV-1 proviral DNA with a frameshift in *env* and encoding GFP

in place of *nef*) and the pSVIIIenv plasmid, encoding the clade B 89.6 Env protein. Supernatant containing reporter virus was collected 48 hrs after transfection, clarified by centrifugation and 0.45μm filtration, and stored at –80°C before use. To perform the assays, human PBMC were incubated with reporter virus in the presence or absence of antibody. The neutralizing antibody responses were measured by the percent reduction of GFP positive human PBMC as compared to the numbers in controls with pre-immunization serum.

One set of neutralization data is depicted in Fig. 9. The strength of neutralizing antibody was ranked at three levels: high (above 80% reduction in GFP positive human PBMC), moderate (above 60% reduction) and low (above 40% reduction). The DNA + protein vaccination approach was highly effective in inducing neutralizing antibody responses against virus expressing Env-GFP of isolate 89.6, a primary HIV-1 virus. All animals except the control animal produced low levels of neutralizing antibody after the DNA vaccination and prior to the protein administration. One animal that received a monovalent vaccination produced low (approximately 50%) levels of neutralizing antibody. A second animal in the monovalent group produced moderate (approximately 75%) levels. Animals that received the 3-valent vaccination produced antibody with high or nearly high (approximately 78%) levels of neutralization. The 8-valent group produced low levels of approximately 40% neutralization. The control rabbit (C10-1), which received a single Env protein immunization after four inoculations of empty DNA vector produced no detectable neutralizing antibody after DNA vaccination, and demonstrates a very low (approximately 10%) neutralizing antibody response after protein administration.

Further analyses of these rabbit sera against additional primary clade B HIV-1 viruses also showed positive neutralizing activities (Fig. 10 to 15). Figs. 9 to 12 show that the DNA prime/protein boost approach using polyvalent formulation was highly effective in inducing neutralizing antibody responses against a number of primary HIV-1 clade B isolates. In these assays, neutralizing antibody responses were measured by the percent reduction of p24 positive PBMC as compared to the numbers in control with pre-immunization serum by a FACS based assay (Mascola, et al., 2002 J. Virol. 76, 4810-4821).

As shown in Figure 10, significant levels of neutralizing antibody responses were present at the end of DNA priming. Sera from mice that received the 3-valent and 8-valent DNA + protein vaccination protocols exhibited high (80%$^{+}$) levels of neutralizing activity

toward the primary HIV-1 isolate, SF162, after one and two protein boosts. Peak levels of neutralizing activity were reached after the first protein boost. High levels of neutralizing activity were obtained in sera of the animal receiving two boosts of an 8-valent protein vaccination (without DNA vaccination).

For Ba-L (Figure 11) and JRCSF (Figure 12), protein boosts were effective in generating higher levels of neutralizing activities than with DNA priming alone. As shown in Figures 11 and 12 high levels of neutralizing activity to primary HIV-1 strain Ba-L and JRCSF, respectively, were obtained in animals receiving the 3-valent vaccination protocol. The 8-valent group showed levels of approximately 60-80% after two protein boosts. With the conditions used, an 8-valent env DNA formulation did not result in a more robust antibody response than that elicited by the 3-valent formulation. It took one protein boost for the 3-valent group to reach the peak neutralizing antibody level while a second protein boost was needed for the 8-valent group. This finding is consistent with the solid phase binding antibody analysis results as shown in the examples below.

Serum from the control animal, 10-1, did not show any neutralizing activity after DNA priming. This animal only received empty DNA vector. However, the serum did show some low level neutralizing activities after one or two protein boosts. The levels of neutralizing activity were much lower than the DNA + protein approach, supporting the observation that DNA priming is very useful for the rapid induction of neutralizing antibody responses by 1-2 protein boosts, especially against the primary HIV-1 isolates, which are often difficult to neutralize.

Additional neutralization assays were conducted to examine whether these rabbit sera also neutralized primary viral isolates representing other HIV-1 clades. Fig. 13shows data obtained after the second protein boost in animals receiving monovalent and polyvalent injections. After two protein boosts, several immunized rabbit sera (dilution 1:5) showed positive neutralizing activities against HIV-1 DJ263 (clade A) and TV1 (clade C) subtypes.

In summary, these data show that the DNA prime/protein boost vaccination modality was effective in inducing neutralizing antibody responses against primary HIV-1 isolates across several subtypes.

Example 10: Rabbit Anti-Env IgG Responses with Codon Optimized Env Gene Inserts in Formulation DP6-001

The immunogenicity of the codon optimized *env* and *gag* DNA vaccine components and the Env protein boost components to be used in the DP6-001 vaccine was examined. Rabbits were immunized with DNA components of DP6-001 vaccine by gene gun, ID or IM inoculation followed by gp120 protein boost by IM route so that anti-Env antibody responses could be compared. The amino acid sequences of the proteins used in the DP6-001 protein boosts are shown in Figure 14, with the sequences aligned to each other. In previous rabbit studies, only non-codon optimized DNA vaccines were used.

For data presented in the previous studies, DNA plasmids were delivered by a gene gun immunization method. The study described in this example demonstrates the immunogenicity of DNA vaccines by intramuscular (IM) and intradermal (ID) injections.

Female rabbits were immunized with DNA vaccines expressing five primary HIV-1 Env antigens and one primary HIV-1 Gag antigen followed by two protein boosts including five primary gp120 antigens. The details of the immunization protocols are shown in Table 8. The DNA and protein components used in this study are the same as in the DP6-001 formulation. Each group of New Zealand White rabbits (two per group) received three DNA immunizations at weeks 1, 5, and 13, and two protein boosts at weeks 21 and 29. Animals received DNA immunization either by a gene gun (GG), IM or by ID injection. Proteins were formulated in QS-21 adjuvant and immunized by IM route. The total dose of immunogens for each immunization is listed in Table 8.

Table 8: Design of Rabbit Groups Immunized with Codon Optimized DNA Vaccines

Groups	DNA priming	DNA Route	Total DNA Dose Per Immunization	Protein Boosting	Total Protein Dose Per Immunization
C30	Env (A, B, Ba-L, Czm, E) + Gag (Czm)	Gene Gun	36 μg (6 μg per individual DNA)	A, B, Ba-L, Czm, E	100 μg (20 μg per protein)
C31	Env (A, B, Ba-L, Czm, E) + Gag (Czm)	IM	600 μg (100 μg/DNA)	A, B, Ba-L, Czm, E	100 μg (20 μg per protein)
C32	Env (A, B, Ba-L, Czm, E) + Gag (Czm)	ID	600 μg (100 μg per DNA)	A, B, Ba-L, Czm, E	100 μg (20 μg per protein)

ELISA data showing anti-Env IgG responses in NZW rabbits that received DNA priming by different routes (gene gun, IM or ID) is depicted in Figures. 15A-E. Figures 15A, 15B, 15C, 15D, and 15E depict responses against HIV-1 A, HIV-1B, HIV-1 Czm, HIV-1 E, and HIV-1 Ba-L isolates, respectively. Overall, the polyvalent formulation was able to induce broad antibody responses recognizing all five primary Env antigens.

While gene gun immunization remains the most effective approach in priming anti-Env antibody responses, both IM and ID routes were able to prime the animals and induced anti-Env IgG responses soon after one protein boost. It appears that ID had higher variations in antibody responses than the IM injection group. The antibody responses remained at relatively high level for more than 8 weeks after the last boost.

These data show that the DP6-001 formulation is immunogenic in NZW rabbits. Both IM and ID routes are effective in priming the anti-Env antibody responses, similar to the gene gun approach. Protein boosts are highly effective to bring the antibody responses to peak level primarily in animals immunized with DNA by ID and IM routes. Inclusion of a *gag* DNA construct did not appear to interfere with the immunogenicity of the polyvalent Env-expressing DNA plasmids.

Example 11: Serum Antibody Titers Elicited by a Repeat-dose Intramuscular, Intradermal, or Intramuscular and Intradermal DP6-001 Vaccine During a Toxicity Study in Rabbits

The antibody responses elicited by repeated administration of DP6-001 vaccine in New Zealand White rabbits were evaluated to examine whether sera from rabbits that participated in a toxicity study had anti-Env and anti-Gag antibodies reactive to DP6-001 vaccine immunogens. For the DNA immunization phase, animals (at least five/sex/group total) were immunized four times every four weeks intramuscularly for a total animal dose of 7.2 mg per immunization (in 1.2 ml of diluent per DNA) or intradermally for a total animal dose of 3.6 mg per immunization (0.6 mg per DNA) of polyvalent DNA vaccine. Control animals were alternated (between dates of injection) between intradermal and intramuscular injections with the saline control. For the protein boost phase animals were immunized three times every four weeks intramuscularly for a total animal dose of 0.375 mg per immunization (0.075 mg per gp120). Each protein dose contained 0.05 mg of the adjuvant, QS-21, and 30 mg of excipient, cyclodextrin. QS-21 is a saponin adjuvant (a 3,28-O-bisglycoside quillaic

acid) that can be obtained in high purity from Quillaja saponaria Molina extracts (Kensil, et al., 1998, Dev Biol Stand., 92:41-7). Sera were collected from each rabbit fourteen days after either four DNA or four DNA and three protein immunizations, and assayed by ELISA for antibodies to pooled gp120 (immunogens in DP6-001) and to Gag protein. Sera from control animals were also assayed for background reactivity.

Fig. 16shows anti-Env antibody titers in male and female rabbits immunized with 7.2 mg of DNA by IM route or 7.2 mg of DNA by IM route followed by 0.375 mg of gp120 boost intramuscularly. Fig. 17 shows anti-Env antibody titers in male and female rabbits immunized with 3.6 mg of DNA by ID route or 3.6 mg of DNA by ID route followed by 0.375 mg of gp120 boost intramuscularly. Fig. 18 depicts anti-Gag antibody titers in male and female rabbits immunized with 7.2 mg of DNA by IM route or 7.2 mg of DNA by IM route followed by 0.375 mg of gp120 boost intramuscularly. Fig. 19 shows anti-Gag antibody titers in male and female rabbits immunized with 3.6 mg of DNA by ID route or 3.6 mg of DNA by ID route followed by 0.375 mg of gp120 boost intramuscularly.

DNA delivered by both IM and ID routes were able to elicit strong antibody response in rabbits against both gp120 and Gag proteins. Antibody titers to gp120 were boosted significantly following gp120 protein boost. As expected, titers to Gag protein were not affected since the protein boost did not contain Gag protein. No significant difference in antibody titers was observed between groups receiving 3.6 or 7.2 mg of DNA. Sera from control animals did not show any reactivity with either gp120 or Gag protein (not shown).

Both DNA and gp120 protein immunizations of DP6-001 vaccine performed during the toxicology study elicited strong antibody response in rabbits. DNA immunizations had a very strong priming effect, which was significantly boosted following gp120 protein immunizations.

Examples 12 and 13: Immunogenicity of Polyvalent DNA Vaccines Encoding Codon Optimized env and gag Genes Followed by gp120 Protein Boost in Nonhuman Primates

Two studies were conducted in nonhuman primates to evaluate immunogenicity of polyvalent DNA prime gp120 protein boost vaccines. In the first study (referred as DNA wt/ Protein Study 1), immunogenicity of five DNA vaccines encoding wild type gp120 (B715, Ba-L, Czm and E) and gag (pNL4-3) genes were delivered either by ID or gene gun route.

The protein boost consisted of gp120 from clades B715, Ba-L, Czm and E. This study revealed that DNA delivered by gene gun favored antibody over CMI response whereas DNA delivered by ID route had measurable CMI response. Antibody responses were markedly enhanced following gp120 boost in both ID and gene gun groups. To elicit both CMI and antibody responses, a second study (referred as DNA opt/ Protein study 2) was conducted where immunogenicity of DP6-001 vaccine containing codon-optimized gp120 genes from A, B715, Ba-L, Czm and E isolates and Czm gag gene was examined. In this study DNA was delivered by either ID or IM route.

Example 12: DNA wt/Protein-Study 1 to Investigate the Immunogenicity of a Polyvalent Combination Vaccine of DNA Encoding four Wild Type gp120 Env Proteins and a Gag Protein and Boosts With Four gp120 Proteins in Rhesus Macaques

The experiments in this example were undertaken to examine immune response in rhesus macaques elicited by priming with polyvalent DNA vaccines encoding a Gag protein and four Env proteins followed by boosting with four gp120 proteins homologous to the DNA vaccines. Six rhesus macaques (male) were included in this study. The polyvalent vaccine formulation tested in this study had one Gag and four Env antigens unlike the DP6-001 formulation, which has one Gag and five Env antigens. Animals were immunized four times with a mixture of five DNA plasmids (four plasmids encoding wild type *env* genes from clade B Ba-L, clade B B715, clade C Czm and clade E 976; and one plasmid encoding HIV-1 clade B *gag* gene) in saline. Three animals (961L, 963L and 969L) were immunized with DNA by gene gun, whereas the other three animals (971L, 974L and 975L) received DNA by ID route. Each animal was then boosted with purified gp120 from four isolates representing B, Ba-L, Czm and E two times by IM route. For each ID immunization, 500 μg of each plasmid DNA (2.5 mg total) was injected separately. For gene gun inoculation, 20 μg of each DNA (100 μg total) was delivered. Each animal received 75 μg of each gp120 (300 μg of pooled gp120) formulated in 100 μg of QS-21 adjuvant in PBS. Animals were immunized with DNA on weeks 0, 6, 12 and 18 followed by protein boosts on weeks 24 and 32. Serum was collected at designated times, generally two weeks after each immunization.

Serum antibody titers to all five envelope and the Gag proteins following each immunization of DNA and protein were assayed by ELISA (Figure 20). These results

clearly demonstrate that DNA delivered via gene gun elicited higher antibody response against gp120 and Gag proteins than DNA administered by ID inoculation, and antibody titers increased progressively following each DNA immunization. However, boosting of DNA primed animals with a single gp120 protein enhanced antibody titers markedly in both groups of animals to a comparable level. A slight increase in antibody response was noted following the second gp120 boost. As expected, no change in anti-gag antibody titers was noted following gp120 immunization (Figure 20E).

To determine whether antibodies elicited by this vaccine are functional, sera collected after the fourth DNA and two protein boost were assayed for neutralization of HIV-1 and SHIV isolates encoding *env* genes homologous to gp120 genes of DP6-001 vaccine. These viruses include both $SHIV_{Ba\text{-}L}$ and primary $HIV\text{-}1_{Ba\text{-}L}$ isolates encoding Ba-L *env* gene, $HIV\text{-}1_{Czm}$ encoding Czm *env* gene, and $HIV\text{-}1_{B715}$ encoding B715 *env* gene. Sera obtained from macaques after four DNA immunizations and gp120 boost neutralized $SHIV_{Ba\text{-}L}$ (Figure 21). Four DNA immunizations did not elicit neutralizing activity against a $SHIV_{Ba\text{-}L}$ isolate (Figure 21A). However, sera from the immunized animals harvested after each gp120 boost were able to inhibit $SHIV_{Ba\text{-}L}$ infection (Figures 21B and C). Neutralization titers of sera from these animals harvested after protein boost against a number of homologous and a few heterologous HIV-1 isolates are presented in Table 9A. Percent inhibition of infection by the sera is presented in Table 9B. Sera collected after four DNA inoculations did not neutralize any of these isolates (data not shown). However, a few of these isolates were neutralized by sera after protein boost.

Table 9A: Neutralization Titers of Serum from Immunized Animals Against HIV-1/SHIV Isolates

Animal Number	Neutralization Titer					
	HIV-1 Clade B				HIV-1 Clade C	
	SHIVBa-L	MN	89.6	B715	93MW160	931N101
961L	23	41	6	<5	<5	<5
963L	41	100	6	<5	<5	<5
969L	24	48	15	<5	<5	<5
971L	27	54	8	5.5	<5	<5

974L	78	100	8	10	<5	<5
975L	17	51	7	<5	<5	<5

Serum collected after 2 Protein boosts were assayed. Neutralization titers are calculated based on the dilution of immune serum inhibiting 50% of infection compared to untreated controls.

Table 9B:

Animal Number	% Inhibition of Infection					
	HIV-1 Clade B					
	Ba-L	SF162	ADA	5768	515	PVO
961L	80	63	0	0	0	0
963L	77	87	0	0	0	0
969L	16	81	10	0	0	0
971L	67	66	0	0	0	0
974L	89	93	0	0	0	0
975L	47	76	0	0	0	0

Serum collected after 4 DNA and 2 Protein boosts were assayed. Each serum was tested at 1:16 dilution for neutralization of indicated HIV-1 isolates using human PBMC targets. Percent inhibition was based on the degree of infection observed in the presence of immune serum compared to untreated controls

In summary, immunization of macaques with polyvalent DNA vaccines encoding four *env* genes and a *gag* gene primes the immune system significantly. Protein boosts are highly effective in eliciting a broad antibody response. This antibody response was able to neutralize homologous, and to lesser extent, heterologous primary HIV-1 isolates.

Example 13: DNA opt/Protein-Study 2: Immunogenicity of DP6-001 Vaccine in Rhesus Macaques

Humoral and cellular immune responses elicited by DP6-001 vaccine in rhesus macaques were examined. Six (five male and one female) rhesus macaques participated in this study. Animals were immunized with a mixture of six DNA plasmids: five plasmids

encoding codon optimized env genes from clade A, B Ba-L, clade B B715, clade C Czm and clade E 976, and one plasmid encoding HIV-1 clade C gag gene, in saline four times. Three animals (51M, 978L, 980L) were immunized with DNA by IM route and three animals (991L, 997L, 998L) received DNA by ID route. Each animal was then boosted with purified gp120 from five isolates representing A, B, Ba-L, Czm and E two times by IM route. For each immunization, 500 μg of each plasmid DNA (3 mg total) was pooled and suspended in a total volume of 2 ml saline. Each animal received 3 mg of total DNA either by IM or by ID route. For ID immunization DNA was delivered into 19 sites (100 μl per site). For IM inoculation, DNA was delivered into 4 sites (500 μl per site). Animals received 375 μg of pooled gp120 (75 μg of each gp120) in 100 μg of QS-21 adjuvant in 1 ml of PBS. Animals were immunized with DNA on weeks 0, 6, 12 and 18 followed by protein boost on weeks 24 and 32. Serum was collected approximately two weeks after each immunization. Sera were collected up to week 49 for a binding antibody assay.

Antibody titers to all five envelope proteins following each immunization of DNA and protein were assayed by ELISA and the results are shown in Figure 22. These results demonstrate that DNA delivered via the IM route elicited a higher antibody response against at least against three out of five envelope proteins as compared to DNA delivered via an ID route, and antibody titers increased progressively following each DNA immunization. However, boosting of DNA primed animals with gp120 protein enhanced antibody titers markedly in both groups of animals to a comparable level. Antibody levels decreased slightly following the second protein boost with progressive drop over time. Titers of anti-Gag antibodies were low in each animal during both DNA and protein immunization phase (data not shown).

To determine whether antibodies elicited by the DP6-001 vaccine are functional, sera collected after the fourth DNA administration and the protein boost were assayed for neutralization of a few HIV-1 and SHIV isolates encoding env genes homologous to gp120 genes of the DP6-001 vaccine. These viruses include both $SHIV_{Ba\text{-}L}$ and primary $HIV\text{-}1_{Ba\text{-}L}$ isolates including a Ba-L env gene, $HIV\text{-}1_{Czm}$ a Czm env gene and $HIV\text{-}1_{B715}$ including a B715 env gene. Neutralization of both cell-free and cell-to-cell transmission of HIV-1/SHIV by the hyperimmune sera was assayed. For assays with cell-free virus, heat inactivated serum was incubated at 37°C with cell-free virus and the virus/serum mixture was

subsequently used to infect a U373 cell line containing the reporter gene β-galactosidase. Neutralization of cell-to-cell transmission of HIV-1 was conducted by a syncytium inhibition assay in which chronically infected cells were cocultured with uninfected cells in the presence of the sera. Coculturing of cells induces syncytium formation which was inhibited by the neutralizing sera. Each neutralization assay included controls where infection assays were conducted with either no serum or pre-immune or normal rhesus serum.

Figure 23 demonstrates neutralization of a $SHIV_{Ba-L}$ isolate by sera from macaques after four DNA immunizations and gp120 boost. Four DNA immunizations did not elicit neutralizing activity against $SHIV_{Ba-L}$ isolate. However, sera from the immunized animals collected after a gp120 boost were able to inhibit $SHIV_{Ba-L}$ infection. Serum collected after four DNA inoculations did not neutralize any of these isolates (data not shown). However, these homologous HIV-1 isolates were neutralized by sera after a protein boost. Neutralization titers of serum from these animals after the first protein boost against a number of homologous HIV-1 isolates are presented in Table 10. Sera after first protein boost were also tested for neutralization of a number of heterologous HIV-1 isolates, and the results are shown in Table 11. Additional neutralization assays were also conducted with serum collected after 4 DNA and first and second protein boosts against a broad range of primary HIV-1 isolates from clades A, B, C and E and the results are shown in Table 12.

Table 10: Neutralization Titers of Serum from Immunized Animals Collected after First Protein Boost Against Homologous HIV-1/SHIV Isolates

	Clade B	Clade C	Clade B
Animal	$SHIV_{Ba-L}$[1]	$HIV\text{-}1_{Czm}$[1,2]	$HIV\text{-}1_{B715}$[1,2]
51M	**82**	**83**	**>1280**
978L	**37**	**61**	**>1280**
980L	**<10**	**38**	**89**
991L	**>160**	**44**	**952**
997L	**41**	**46**	**840**
998L	**>160**	**54**	**>1280**

[1]Neutralization titers are calculated based on the dilution of immune serum inhibiting 50% of compared to untreated controls.

[2]Neutralization of HIV-1_{Czm} and HIV-1_{B715} were assayed by cell-to-cell transmission assay where CEM cells chronically infected with HIV-1_{Czm} or HIV-1_{B715} were cocultured with uninfected CEM cells in the presence of immune serum and syncytia were scored after 48 hrs.

Table 11: Neutralization Titers of Serum from Immunized Animals Collected after First Protein Boost against Heterologous HIV-1 Isolates

Animal	MN clade B[1]	SF162 clade B[1]	Ba-L Clade B[2]	Bx08 clade B[2]	6101 clade B[2]	92RW020 clade A[2]	92RW020 clade A[2]	Dul 79 clade C[2]	CM244 clade E[2]
51M	217	717	55	72	30	37	18	21	34
978L	248	361	6	64	0	10	0	22	19
980L	50	463	70	75	29	20	20	20	18
991L	236	407	54	49	0	15	30	0	0
997L	94	321	36	58	0	0	0	0	0
998L	2455	4632	72	91	13	37	26	28	18

[1]Neutralization titers are calculated based on the dilution of immune serum inhibiting 50% of compared to untreated controls.

[3]Each serum was tested at 1:15 dilution for neutralization of indicated HIV-1 isolates using an indicator cell line. Percent inhibition was based on the degree of infection observed in the presence of immune serum compared to untreated controls as measured by chemiluminescence output.

Table 8-10: Neutralization of Primary HIV-1 Isolates by the Serum of Immunized Animals Collected after 4 DNA and First and Second Protein Boosts

Animal	Immune Serum	Neutralization of HIV-1 (% Inhibition of Infection)									
		Clade B				Clade C			Clade A		Clade E
		SF162	JRCSF	ADA	BAL	TV1	DU151	S007	DJ263	RW020	CM235
51M	Post 4 DNA	24	14	31	-3	5	2	-13	15	-50	-58
	Post-1 Protein	94	46	47	27	74	2	-9	50	14	-66
	Post-2 Protein	86	41	33	20	60	25	-20	45	2	-136
978L	Post 4 DNA	33	29	18	-34	21	2	-8	35	-26	-19
	Post-1 Protein	79	33	39	7	18	11	-7	40	-37	-61
	Post-2 Protein	64	81	28	8	-44	9	-16	51	-13	-130
980L	Post 4 DNA	12	0	8	-18	-16	15	25	36	-50	-21
	Post-1 Protein	62	23	32	1	41	22	8	56	-7	-3
	Post-2 Protein	37	-17	19	-18	-47	30	33	54	-29	-1
991L	Post 4 DNA	-37	-34	33	-37	-34	-9	-35	-21	-17	-68
	Post-1 Protein	76	32	59	3	-34	-2	-23	26	-57	-51
	Post-2 Protein	44	13	46	-23	-99	-31	-31	25	-101	-153
997L	Post 4 DNA	29	-6	32	-30	-28	-28	2	19	-99	-7
	Post-1 Protein	87	33	52	6	-3	15	12	57	-90	-25
	Post-2 Protein	58	0	36	-32	8	15	23	62	-79	-54
998L	Post 4 DNA	51	0	20	-31	-9	-10	-9	16	-67	-68
	Post-1 Protein	96	82	63	59	67	20	3	68	16	-90
	Post-2 Protein	85	76	46	-11	-2	5	2	56	-33	-122

Serum was tested at 1:5 dilution. Assay was conducted as described elsewhere (Mascola, et al., 2002, J. Virol. 76, 4810-4821).

The anti-Gag specific CMI response elicited by DP6-001 vaccine was assayed by ELISPOT for the production of IFN-γ. In this assay Gag peptide pools each containing six 15-mer peptides with 11 amino acid overlaps were used and two consecutive peptide pools were mixed for stimulation. Gag sequences were from an HIV-1 HXB-2 isolate. As shown in Fig. 24, a number of animals had anti-Gag specific positive ELISPOT responses after four DNA immunizations, with ID inoculation appearing to be more effective than IM inoculation in inducing a cell mediated immune response.

The anti-Gag specific CMI response elicited by wild type and codon optimized *gag* vaccines (Study 1 and Study 2) was also compared. As shown in Fig. 25, immunization of macaques with a codon-optimized *gag* gene elicits significantly higher ELISPOT response compared to DNA encoding wild type *gag* gene.

Anti-Env specific CMI responses elicited by the DP6-001 vaccine against Ba-L and clade E envelopes were assayed by ELISPOT for the production of IFN-γ. The results are shown in Fig. 26. Although a weak ELISPOT response was elicited in animals following four DNA immunizations, boosting of DNA primed animals with the polyvalent gp120 proteins markedly enhanced such response.

Immunization of macaques with DP6-001 DNA vaccines significantly primes the immune systems. Protein boosts are highly effective in eliciting a broad antibody response. CMI responses against the Gag antigen as measured by ELISPOT assay was observed following DNA immunization primarily by ID route. Taken together these results demonstrate that immunization of macaques with the DP6-001 vaccine elicits CMI and a broad binding antibody response, which is able to neutralize a number of HIV-1 isolates.

Example 14: DP6-001 - 63-day Repeat-Dose Intradermal or Intramuscular Biodistribution and Integration Study in New Zealand White Rabbits

The biodistribution of DNA over a course of 64 days following single immunization of DNA via IM or ID route was examined. A total of 54 rabbits (27/sex) were used in this study. The animals were divided equally into three groups. Animals were initially accepted into the randomization pool based upon body weight and physical examination. They were assigned to study groups using computer-generated random numbers. At randomization, the mean body weight for each group was not statistically different from the control mean. Animals were assigned to three groups as shown in Table 13. Control animals (Group 1) were injected once with saline intradermally and intramuscularly. Group 2 animals were administered intramuscularly with a single dose of 7.2 mg of HIV Vaccine (Plasmid) DP6-001 (2.4 ml total at 1.2 ml per injection site), while Group 3 rabbits received a single immunization of 3.6 mg HIV Vaccine (Plasmid) DP6-001 via an intradermal route (1.2 mls total with 0.12 mls per injection site). The intramuscular dose was equally distributed between 2 injection sites on the left and right thigh muscles (1.2 ml administered per thigh). The intradermal dose was equally distributed between 10 injection sites, located in the dorsal area (approximately 0.12 ml per injection site). All injection sites were shaved and marked. The overall study design is described in Table 13.

Table 13: Design of Study Conducted to Examine Biodistribution of DNA in Rabbits

Group	Test Article	Clinical Dose	Dose Mg	Dates of Dose	Route[2]	Dose Volume[3] mL	Number of Animals	
							Male	Female
1	Saline Control		NA	SDI	IM/ID	2.4/1.2	9	9
2	DNA plasmid	IX	7.2	SDI	IM	2.4	9	9
3	DNA plasmid	IX	3.6	SDI	ID	1.2	9	9

[2]IM: Intramuscular; IM/ID: Intramuscular and Intradermal combination (Intramuscular and Intradermal route of administration will alternate between dates of dosing); ID; Intradermal; NA: Not applicable

[3]Dose Volume is constant regardless of animals' body weights

Cageside observations included observations for mortality, moribundity, general health and signs of toxicity. Clinical observations included evaluation of skin and fur characteristics, eye and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor and behavior patterns.

Six animals (three per sex per timepoint) were sacrificed on study day (SD) 8, 29, and 64 by sodium pentobarbital injection and the following tissues were removed, snap frozen in liquid nitrogen, and stored at –70 ± 10°: blood (prior to euthanasia, ~ 1 mL was collected by puncture of the medial auricular artery into EDTA tubes, inverted several times and transferred into cryovial tubes); ovaries/testes; thymus; heart; lung; liver; gastrointestinal (small intestine section); kidney; spleen; subcutis (at intradermal injection site only); skin at intradermal injection site only (representative sample); intradermal injection site muscle, both sites (representative sample; an extra ~2 g sample was taken on SD 64 for integration analysis); intramuscular injection site muscle, both sites (an extra ~2g sample was taken on SD 64 for integration analysis); contra lateral popliteal or mesenteric lymph node; bone marrow (isolated from the femur); and brain.

All tissues were processed (except skin samples) and analyzed by qPCR for the presence of the plasmids using a Good Laboratory Practice (GLP) validated method for biodistribution.

No test article-related changes in mortality, clinical signs of toxicity, body weights, body weight changes, or food consumption were observed. Biodistribution qPCR (Quantitative Polymerase Chain Reaction) analysis determined that the HIV Vaccine (Plasmid) DP6-001 was present in the muscle and subcutis at the intradermal injection sites and muscle at the intramuscular injection sites. Frequency of findings and copy number were greatest at the SD 8 necropsy and decreased progressively through the SD 29 and 64 necropsies. Only a few sporadic findings were evident in other tissues and these were considered the result of biological variation. Since the plasmid persisted at the intradermal and intramuscular injection sites through SD 64, integration analysis was performed on representative injection site muscle samples. Integration analysis was performed by extracting DNA from sites of administration and performing qPCR to determine if vaccine sequences were present in high molecular weight (i.e., chromosomal) DNA. If this assay tested positive, chromosomal DNA was extracted from the tissue and purified using field inversion gel electrophoresis, and retested for the presence of vaccine DNA. No integration was detected on samples for this study.

In summary, single intramuscular or intradermal injection of HIV Vaccine (Plasmid) DP6-001 in New Zealand White rabbits did not exhibit any obvious signs of toxicity under the study conditions used. The plasmid distributed into the intradermal injection site muscle, subcutis, and the intramuscular injection site muscle without any integration at the injection site.

Example 15: Tolerability and Safety of DP6-001

The tolerability and safety profile of the DP6-001 vaccine formulation was examined in the rabbit model. Since this vaccine formulation contains both DNA and protein components, the potential toxicity of both of these components was examined using the highest dose to be used in the phase I trials, with each animal receiving an additional inoculation compared to the clinical protocol. As proposed in the clinical trial, DNA was delivered either by IM or ID route whereas protein was inoculated by IM route in the toxicology study. Since protein immunization can be formulated with QS-21 adjuvant and with the excipient cyclodextrin, the potential toxicity of QS-21/cyclodextrin mixture was also

examined in an additional arm of the study using the dose to be used in the clinical trial. The salient features of the toxicity study and the overall conclusions are discussed below.

The potential toxicity of a plasmid prime and protein boost HIV Vaccine DP6-001 when administered repeatedly at multiple dose levels by the intramuscular or intradermal route during a 26-week study period to male and female New Zealand rabbits were examined. The rabbit model was selected because it is recommended by FDA for use in vaccine preclinical studies. The intramuscular and intradermal routes of immunization were selected since these are the potential routes for administration to humans. The DNA vaccine component is a mixture of six different DNA plasmids in equal concentrations expressing six different HIV protein variants and was used for the DNA prime phase of the study. The protein component of the DP6-001 vaccine is a mixture of five different proteins in equal concentrations expressing five different HIV protein variants and was used for the protein boost phase of the study. The dose (7.2 mg of pooled DNA) selected for the plasmid immunogens to be delivered intramuscularly were based on expected clinical dose. However, for intradermal immunization, toxicity dose (3.6 mg) of plasmid DNA was three times the proposed clinical dose. The protein dose used in toxicity study was comparable to the clinical dose.

Animals were initially accepted into the randomization pool based upon body weight and physical examination. They were assigned to study groups using computer-generated random numbers. At randomization the mean body weight for each group was not statistically different from the control mean. Animals were assigned to groups as shown in Table 14.

Table 14: Design of Toxicology Study Conducted with DP6-001 Vaccine

Group	Test Article	Clinical Dose[1]	Dose[2] mg	Dates of Dose	Route[3]	Dose Volume[4] ml	Number of Animals	
							Male	Female
1	Saline Control	IX	NA	SD	IM/ID	2.4/1.2	8	8
	PBS Control	IX		SD	IM	1.0		
2	Saline Control	IX	NA	SD	IM/ID	2.4/1.2	8	8

	PBS Control	IX		SD	IM	1.0		
3	QS-21	IX	NA	SD	IM	1.0	10	10
4	DNA plasmid	IX	7.2	SD	ID	2.4	5	5
5	DNA plasmid	3X	3.6	SD	ID	1.2	5	5
	DNA plasmid	IX	7.2	SD		2.4		
	Protein	IX	0.375	SD	IM	1.0	5	5
	DNA plasmid	3X	3.6	SD	ID	1.2	5	5
7	Protein	IX	0.375	SD	IM	1.0	5	5
8	DNA plasmid	IX	7.2	SD	IM	2.4	5	5
9	DNA plasmid	3X	3.6	SD	ID	1.2	5	5
	DNA plasmid	IX	7.2	SD		2.4		
10	Protein	IX	0.375	SD	IM	1.0	5	5
	DNA plasmid	3X	3.6	SD	ID	1.2		
11	Protein	IX	0.375	SD	IM	1.0	5	5

[1]The values supplied are the multiple of the highest expected human clinical dose

[2]Protein to be given with QS-21 at 50 μg and cyclodextrin at 30 mg

[3]IM: Intramuscular; IM/ID; Intramuscular and Intradermal combination (Intramuscular and Intradermal route of administration will alternate between dates of dosing); UDL: Intradermal; NA: Not Applicable

[4]Dose Volume is constant regardless of the animals' body weights

The dose administration scheme for both DNA and protein immunogens are shown in Table 15.

Table 15: Dose Administration Scheme of DNA and Protein Immunogens in Toxicology Study

Route of Administration	**Intramuscular, Intradermal, or combination Intramuscular and Intradermal (alternated between dates of dosing starting with Intramuscular)**
Frequency of Dosing	**Once daily on SD 1, 29, 57, 85, 113, 141, 169 as designated in Table 14**
Dose Volume	**Plasmid IM: 2.4 mL split between two dosing sites Protein IM: 1.0 mL into one site Plasmid ID: 1.2 split**

	between 10 dosing sites
Dose Sites[a]	**Intramuscular: right and left thighs for plasmid and right thigh for protein** **Intradermal: dorsal scapular area**
Equipment	**Intramuscular: 23 gauge, 5/8 inch needle with 3mL syringe** **Intradermal: 0.5 mL syringe with 27 gauge 1/8 inch needle**
Dosing Conditions	**Formulations were maintained on wet ice until administered**

a – Injections were administered at a shaved/marked site. The sites were re-shaved and re-marked as needed.

IM – Intramuscular ID – Intradermal

New Zealand White rabbits (5/sex/group minimum) received intramuscular (2.4 ml dose volume, resulting in a 7.2 mg/animal dose) or intradermal (1.2 ml dose volume, resulting in a 3.6 mg/animal dose) administration of the DP6-001 HIV DNA vaccine or saline control on Study Day (SD) 1, 29, 57, and 85. For the protein boost phase of the study, rabbits received intramuscular (1.0 ml dose volume, resulting in a 0.375 mg/animal dose) administration of the DP6-001 HIV protein vaccine, Phosphate Buffered Saline control, or QS-21 and cyclodextrin adjuvant control (50 μg and 30 mg per injection, respectively) on SD 113, 141, and 169. Animals were then necropsied on SD 87 (acute DNA necropsy), SD 99 (recovery DNA necropsy), SD 171 (acute protein necropsy) or SD 183 (recovery protein necropsy). Parameters evaluated included mortality, clinical observations, draize observations, body weights, food consumption, clinical pathology, organ weights, gross pathology, and histopathology. Binding antibody titers elicited by DNA and protein immunizations were also measured.

No test article-related changes in mortality, clinical observations, body weights, food consumption, organ weights, clinical pathology findings, gross observations, and histopathology were observed. Both DNA priming and protein boosts elicited strong serum antibody response as measured by ELISA. An increased frequency of recoverable Draize findings at the intradermal DNA vaccine injection site was observed. Some specific observations made under each parameter examined are given below.

Mortality and clinical observations: Treatment with HIV Vaccine DP6-001 did not result in mortality, and had no effect on clinical observations or cageside observations. One

Group 3 male had an abrasion on the nose on SD 64 and 78. One Group 10 male had swelling of the scrotum on SD 92 that was not observed in any other Group 10 or Group 6 animals. One male in each of Groups 3, 4, 5, 7, 9, and 10 was observed as being thin at various times during the study. These observations resolved within three weeks or less of the first observation, and the low incidence suggested a lack of any test article effect. One Group 1 male animal was found to have abrasions/abscesses on the front and rear paws. Since this was a control animal, these finding were not considered test article-related. Due to these abscesses, the animal had appetite loss and was observed as pale during this period. Several animals had intermittent observations of lacrimation throughout the study.

Draize Observations: Intramuscular treatment with HIV Plasmid Vaccine DP6-001 had no effect on dermal observations. For the Intramuscular injections of plasmid (treated sites 1 and 2), primarily minimal erythema and edema scores were seen, with the exception of a few mild scores. Since these observations were also seen in the control groups (Groups 1 and 2), they were not considered test article-related and were attributed to the injection procedure.

Intradermal treatment with HIV Plasmid Vaccine DP6-001 had an effect on dermal observations. For the intradermal plasmid administration (treated site 3), erythema and edema scores for the controls (Groups 1 and 2 on SD 29 and 85) reached mild levels of intensity. For groups 5, 7, 9, and 11, the intensity and frequency of observations were increased as compared to the controls, suggesting a test article-mediated increase in dermal reactivity. However, while test article mediated, these observations did recover with time.

For Intramuscular injection with HIV Protein Vaccine DP6-001, minimal to mild findings for erythema and edema were evident in the control (Groups 1-3) and protein immunized groups (Groups 6, 7, 10, and 11). Since the findings were comparable between control and protein immunized groups, these were not considered to be test article related and were instead attributed to the injection procedure.

Body weight and food consumption: No treatment-related effects on body weight or body weight gains gains were observed in any groups. Further there were no treatment-related effects on food consumption in any group.

Clinical Pathology: There were no apparent test article-related effects due to repeated multiple dose levels of HIV vaccine DP6-001 in the rabbit. The higher globulin observed in

some groups on SD31 and 87 were very slight and may be due to antibody production, but were most likely due to individual animal variation. All other minor changes were attributed to individual animal variation, and they had no biological significance.

Hematology, clinical chemistry, coagulation: No statistical differences between control and immunized groups were noted and all differences were due to individual animal variation.

Gross Pathology: There were no treatment-related effects on gross pathology. Since there were no histopathological correlates, no dose response was observed, and most of the findings seen were also seen in the control animals, these findings were not considered test article related.

Organ Weights: There were no treatment-related changes in organ weights in any group. Statistically significant changes in organ weights, organ to body weight, and organ to brain weight ratios include: a decrease in the brain weight of the Group 4 females on SD 87, decrease in the thymus weight of the Group 3 females on SD 171, decreases in the adrenal, heart, and spleen weights of the Group 3 females on SD 183, and a decrease in the spleen to body weight ratio and heart to brain weight ratio of the Group 3 females on SD 183. These changes were not considered to be test article-related since no obvious pattern was observed and they involve changes in tissue weights or ratios in the Group 3 adjuvant control animals as compared to the Group 1 or 2 saline control.

Histopathology: There were no treatment-related effects on histopathology in any Groups.

Inflammatory responses, foreign material, and occasional hemorrhage observed at injection sites were considered related to the dosing procedure. There were no histopathological changes considered to be toxic effects of the administration of the test article(s) or vehicle(s). Findings at injection sites included inflammatory cellular infiltration or inflammation at minimal and mild severities, with infrequent hemorrhage and comparatively low incidence of focal refractive deposits of foreign material. These findings occurred in both control and treated rabbits, with no apparent treatment group-related differences and all were considered to be related to dosing procedures.

In kidneys, nephropathy (recognized as scattered microfoci of tubular epithelial cell regenerative and/or degenerative changes with tubular disorganization, hyperchromatic cells,

tubular cell vacuolization, and the collection of pale flocculent material and cellular debris in lower segments of collecting tubules) was seen at minimal severity across all groups. Multifocal tubular mineralization or tubular cell vacuolization occurred separately or in combination with nephropathy. These renal findings are typical of spontaneous pathology in kidneys of New Zealand White rabbits of this age; they showed no apparent relationship to administration of the test article(s).

Fatty change and vacuolation of hepatocytes as well as focal mixed cell or mononuclear cell infiltrate(s) in the liver correlated microscopically with enlargement and discoloration observed grossly. These hepatic findings occur spontaneously in rabbits, and were seen without apparent relationship to dosage.

The sporadic occurrence of hemosiderin deposits in the spleen, focal inflammatory responses in the lung, fatty change in the subepicardium of the heart, and other low incidence sporadic findings in other organs showed no apparent relationship to dose and were considered unrelated to test article administration.

Therefore, under these study conditions, repeat intramuscular administration of HIV Vaccine DP6-001 to New Zealand White rabbits did not exhibit any specific signs of systemic toxicity but resulted in reversible Draize observations at the injection site.

Example 16: Phase I Clinical Study of DP6-001

Human clinical trials are conducted for the purpose of determining safety of a vaccine and for determining efficacy of a vaccine. To determine safety, normal volunteers are immunized with the vaccine. The incidence of side effects is noted. To determine efficacy, NIH established protocols are followed. High-risk population (e.g., drug users, populations with high-risk sexual activity, populations in which the incidence of HIV is high). To test a high risk population, the incidence of HIV infection in the negative control group who are immunized with a DNA vaccine containing the vector alone is compared to the incidence of HIV infection in the test group receiving the polyvalent DNA vaccine containing primary isolate sequences (e.g., sequences of gp120, gp140, gp160 and/or gp41). A double blind trial is conducted. The immunization regimen is, for example, three DNA vaccine immunizations by gene gun, each administered a month apart. Sera are drawn during the regimen to monitor immune status by experiments such as described in Examples 2-4, above. Additionally, cell-

mediated immunity (CTL response) is tested in human patients by isolating PBMCs followed by in vitro functional testing of these cells as described for splenocytes in Example 4, above. The presence of neutralizing antibodies in the patient's sera is then tested as described in Example 3, above. Infection by HIV is tested and statistical analysis is done to determine if the incidence is significantly different between control and test groups.

A phase I clinical study to assess DP6-001 is conducted as follows. The objectives of the study are to assess the safety of multiple dosing levels of DP6-001, to assess the ability of DP6-001 to induce humoral immune responses to vaccine components, and to assess the ability of DP6-001 to induce cell-mediated immune responses.

Approximately 36 human subjects participate in the study. These subjects are healthy, HIV-uninfected adult volunteers of 18-55 years of age. They are at low or minimal risk for HIV infection as defined by HVTN Risk Status. They have no history of previous experimental HIV vaccine inoculations.

Each participant receives one of three dose regimes in which DP6-001 is administered via ID or IM routes. Administration is randomized, with a rising DNA component, multiple doses, with a follow-on protein vaccine boost. One test program of administration to humans is as follows: administer approximately 50 μg/kg of the DP6-001 DNA composition at week 0, week 4, and week 12, (i.e., 3 doses per person, approx. 2.5 mg dose for a person of 50 kg); and administer 7 μg/kg of the DP6-001 protein composition at week 20 and week 28.

To assess the ability of DP6-001 to induce humoral responses to vaccine components, ELISA is performed using a pool of the gp120 glycoproteins used for vaccination. ELISA using HIV-1 Czm Gag protein is also performed. Neutralizing antibody assays against panels of laboratory adapted and primary HIV-1 isolates are performed by HVTN-certified laboratories. Additional solid-phase assays such as Western blots, can be used to further confirm immunity and characterize immune responses and distinguish between vaccination and potential new infection. For example, if the vaccine does not include gp41, the vaccinated subject would not exhibit a response to gp41. Detection of gp41-reactivity in the subject would then be indicative of potential new infection.

To assess the ability of DP6-001 to induce cell-mediated immune responses, IFN-γ ELISPOT assays specific for HIV-1 Gag or Env epitopes can be performed. Bulk culture

cytotoxic T-cell assays and flow cytometric intracellular cytokine staining assays can also be used.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.